FORM PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		22488-710 U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/600521
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/26221	05 November 1999	06 November 1998

TITLE OF INVENTION

A METHOD OF TREATING TUMORS USING FAS-INDUCED APOPTOSIS

APPLICANT(S) FOR DO/EO/US

Jian-Yun DONG, James S. NORRIS

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 37(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☐ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☒ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   c. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
    ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Copy of International Search Report and PCT Publication document.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Unknown 09/600521	PCT/US99/26221	22488-710

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		CALCULATIONS	PTO USE ONL
Search Report has been prepared by the EPO or JPO	$840.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$690.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	$970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 970	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$ 130	

CLAIMS	NUMBER	NUMBER EXTRA	RATE		
Total claims	66 - 20 =	46	46 X $18.00	$ 828	
Independent claims	5 - 3 =	2	2 X $78.00	$ 156	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			$260.00	$	

TOTAL OF ABOVE CALCULATIONS =	$	2,084
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).	$	1,042
SUBTOTAL =	$	1,042
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$	
TOTAL NATIONAL FEE =	$	1,042
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$	1,040
Amount to be: refunded	$	
charged	$	1,040

a. ☐ A check in the amount of $____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 23-2415 in the amount of $ 1,040 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to Deposit Account No. 23-2415. A duplicate of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304-1050
(650) 493-9300

Shirley Chen
SIGNATURE

Shirley Chen
NAME

44,608
REGISTRATION NUMBER

PTO/SB/11 (12-97)
Approved for use through 9/30/00. OMB 0651-0031
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(d))--NONPROFIT ORGANIZATION

Docket Number (Optional)
22488-710

Applicant, Patentee, or Identifier: MUSC Foundation for Research Development

Application or Patent No.:

Filed or Issued:

Title: A Method of Treating Tumors Using FAS-Induced Apoptosis

I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF NONPROFIT ORGANIZATION MUSC Foundation for Research Development

ADDRESS OF NONPROFIT ORGANIZATION 261 Calhoun Street, Suite 305, Charleston, SC 29425

TYPE OF NONPROFIT ORGANIZATION:

☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))

☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)

☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)

I hereby state that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

☒ the specification filed herewith with title as listed above.
☐ the application identified above.
☐ the patent identified above.

I hereby state that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern, or organization having rights in the invention must file separate statements as to their status as small entities and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern, or organization having any rights in the invention is listed below:

☐ no such person, concern, or organization exists.
☒ each such person, concern, or organization is listed below.

GENPHAR, Inc., 871 Low Country Boulevard, Mount Pleasant, South Carolina 29464

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

NAME OF PERSON SIGNING Kenneth J. Roozen, Ph.D.

TITLE IN ORGANIZATION OF PERSON SIGNING Executive Director

ADDRESS OF PERSON SIGNING 261 Calhoun Street, Suite 305, Charleston, SC 29425

SIGNATURE [signature] DATE 7/13/00

PTO/SB10 (12/97)
Approved for use through 9/30/00. OMB 0651-0031
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(c)) -- SMALL BUSINESS CONCERN	Docket Number (Optional) 22488-710
Applicant, Patentee, or Identifier:	GENPHAR, INC.
Application or Patent No.:	
Filed or Issued:	
Title:	A METHOD OF TREATING TUMORS USING FAS-INDUCED APOPTOSIS

I hereby state that I am

~~the owner of the small business concern identified below~~

☒ an official of the small business concern empowered to act on behalf of the concern identified below

NAME OF SMALL BUSINESS CONCERN	GENPHAR, INC.
ADDRESS OF SMALL BUSINESS CONCERN	871 Low Country Boulevard, Mount Pleasant, South Carolina 29464

I hereby state that the above identified small business concern qualifies as a small business concern as defined in 13 CFR Part 121 for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby state that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in:

☒ the specification filed herewith with title as listed above

☐ the application identified above

☐ the patent identified above

If the rights held by the above identified small business concern are not exclusive, each individual, concern, or organization having rights in the invention must file separate statements as to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern, or organization having any rights in the invention is listed below:

☒ no such person, concern, or organization exists

☐ each such person, concern, or organization is listed below.

NAME OF SMALL BUSINESS CONCERN	GEN PHAR, INC
ADDRESS OF SMALL BUSINESS CONCERN	871 LOWCOUNTRY BLVD, MT PLEASANT SC 29464

Separate statements are required from each named person, concern or organization having rights to the invention stating their status as small entities (37 CFR 1.27).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b)).

NAME OF PERSON SIGNING	Steve Hutchinson
TITLE OF PERSON IF OTHER THAN OWNER	~~President~~ CHAIRMAN & CFO
ADDRESS OF PERSON SIGNING	230 Central Park West, #2D, New York, New York 10023
SIGNATURE	DATE 7/13/00

Attorney Docket No. 22488-710

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	Group Art Unit: Not yet assigned
Jian-yun Dong et. al.	Examiner: Not yet assigned
Application No.: Not yet assigned	
Filed: Herewith	
Title: Method of Treating Tumors Using Fas-Induced Apoptosis	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Prior to examination on the merits, please amend the application as indicated below.

Reconsideration is respectfully requested in view of the below amendments and remarks.

Please amend the above-identified application as follows:

IN THE TITLE

Please delete the title and insert –METHOD AND COMPOSITION FOR TREATING TUMORS THROUGH FAS LIGAND-INDUCED APOPTOSIS--.

IN THE SPECIFICATION

Please amend the Specification as follows:

At page 1, line 5, under "BACKGROUND OF THE INVENTION", please insert

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REFERENCES TO PARENT AND CO-PENDING APPLICATIONS

This application claims the priority of U.S. Provisional Application No. 60/107,363 entitled "Method for Treating Tumors Using Fas-Induced Apoptosis", filed on November 6, 1998; and PCT Application No. PCT/US99/26221 entitled "A Method of Treating Tumors Using Fas-Induced Apoptosis", filed on November 5, 1999 and published on May 18, 2000, International Publication No. WO 00/27883. The above applications are hereby incorporated by reference.

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IN THE ABSTRACT

At page 49, please delete the abstract and insert

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ABSTRACT

Methods and expression vectors are provided for inducing death in cells that express an apoptosis-mediating receptor. The method comprises: introducing an expression vector into a group of cells comprising cells that express an apoptosis-mediating receptor, such as a receptor for Fas or Fas-like ligand. The expression vector comprises a polynucleotide sequence encoding an apoptosis-signaling ligand such as Fas or Fas-like ligand whose expression is regulated by a conditional promoter in the vector. The cells into which the expression vector is introduced express the apoptosis-signaling ligand when conditions are suitable to activate the conditional promoter. The expressed apoptosis-signaling ligand induces cell death in those cells which express the apoptosis-mediating receptor through interaction between the apoptosis-signaling ligand and the apoptosis-mediating receptor. The methods and expression vectors can be used for treating tumors in a controlled and site-specific manner.

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IN THE CLAIMS

Please cancel claims 1-46.

Please add the following new claims.

47. A method for inducing death in cells that express an apoptosis-mediating receptor, the method comprising:

introducing an expression vector into a group of cells comprising cells that express an apoptosis-mediating receptor, the expression vector comprising a polynucleotide sequence encoding an apoptosis-signaling ligand whose expression is regulated by a conditional promoter in the vector, the cells into which the expression vector is introduced expressing the apoptosis-signaling ligand when conditions are suitable to activate the conditional promoter, the expressed apoptosis-signaling ligand inducing cell death in those cells which express the apoptosis-mediating receptor through interaction between the apoptosis-signaling ligand and the apoptosis-mediating receptor.

48. The method of claim 47, wherein the apoptosis-mediating receptor is a membrane-bound receptor.

49. The method of claim 48, wherein the membrane-bound receptor is Fas.

50. The method of claim 49, wherein the apoptosis-signaling ligand is capable of binding to Fas.

51. The method of claim 50, wherein the apoptosis-signaling ligand is an antibody that is capable of binding to Fas and signals Fas-mediated apoptosis in cells expressing Fas.

52. The method of claim 50, wherein the apoptosis-signaling ligand is a membrane protein.

53. The method of claim 52, wherein the membrane protein is FasL.

54. The method of claim 49, wherein the group of cells into which the expression vector is introduced comprises a mixture of cells which express Fas and cells which do not express Fas.

55. The method of claim 49, wherein the expression vector is introduced into cells which do not express Fas.

56. The method of claim 49, wherein the expression vector is introduced into cells which do express Fas.

57. The method of claim 49, wherein the expression vector is introduced into cells which do not express Fas and cells which do express Fas.

58. The method of claim 47, wherein the group of cells are contained in a solid tumor.

59. The method of claim 58, wherein the solid tumor is selected from the group consisting of breast, prostate, brain, bladder, pancreas, rectum, parathyroid, thyroid, adrenal, head and neck, colon, stomach, bronchi and kidney tumors.

60. The method of claim 47, wherein introducing an expression vector into the group of cells is performed parenterally, intraperitoneally, intravenously, intraartierally, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery by catheter or stent, subcutaneously, intraadiposally, intraarticularly, intrathecally, or in a slow release dosage form.

61. The method of claim 47, wherein introducing the expression vector is performed by direct injection of the expression vector among the group of cells.

62. The method of claim 47, wherein the expression vector is a plasmid.

63. The method of claim 47, wherein the expression vector is a viral vector.

64. The method of claim 63, wherein the viral vector is selected from the group consisting of adenovirus, adeno-associated virus, vaccinia, retrovirus, and herpes simplex virus vectors.

65. The method of claim 63, wherein the expression vector is an adenoviral vector.

66. The method of claim 47, wherein the conditional promoter is a tissue-specific promoter.

67. The method of claim 66, wherein the tissue-specific promoter is selected from the group consisting of a prostate-specific promoter, a breast-specific promoter, a pancreas-specific promoter, a colon-specific promoter, a brain-specific promoter, a kidney-specific promoter, a bladder-specific promoter, a lung-specific promoter, a liver-specific promoter, a thyroid-specific promoter, a stomach-specific promoter, an ovary-specific promoter, and a cervix-specific promoter.

68. The method of claim 47, wherein the group of cells are prostate cancer cells and the conditional promoter of the expression vector is a prostate-specific promoter.

69. The method of claim 68, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB promoters.

70. The method of claim 47, wherein the conditional promoter is an inducible promoter.

71. The method of claim 70, wherein the inducible promoter is a promoter inducible by tetracycline or doxycycline.

72. The method of claim 70, wherein the inducible promoter is a promoter inducible by steroid.

73. The method of claim 72, wherein the steroid is selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone.

74. The method of claim 47, the method further comprising creating the conditions suitable to activate the conditional promoter.

75. The method of claim 74, wherein creating the conditions suitable to activate the conditional promoter comprises delivering to the group of cells tetracycline or deoxycycline.

76. The method of claim 74, wherein creating the conditions suitable to activate the conditional promoter comprises delivering to the group of cells a steroid selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone .

77. The method of claim 47, wherein the expression vector further comprises a reporter gene.

78. The method of claim 77, wherein the expression vector expresses the reporter gene as a fusion protein with the apoptosis-signaling ligand.

79. The method of claim 78, wherein the reporter gene encodes green fluorescent protein.

80. The method of claim 47, wherein the expression vector further comprises a polynucleotide sequence encoding a regulatory protein.

81. The method of claim 80, wherein the expression vector expresses the regulatory protein as a fusion protein with the apoptosis-signaling ligand.

82. The method of claim 81, wherein the regulatory protein in the fusion protein is a protein that causes tissue-specific localization of the apoptosis-signaling ligand.

83. The method of claim 47, wherein the method is performed *ex vivo* where the group of cells into which the expression vector is introduced are contained in a sample taken from a patient having cancer.

84. The method of claim 47, wherein the method is performed *in vitro* where the group of cells into which the expression vector is introduced are contained in a cell culture.

85. The method of claim 47, wherein the apoptosis-signaling ligand is selected from the group consisting of Bax, Bad, Bak, and Bik.

86. An adenoviral expression vector comprising:

a conditional promoter, and

a polynucleotide sequence encoding a membrane-bound ligand whose expression is regulated by the conditional promoter in the vector, the ligand signaling apoptosis in cells that express an apoptosis-mediating receptor.

87. The vector of claim 86, wherein the membrane-bound ligand is capable of binding to Fas.

88. The method of claim 87, wherein the membrane-bound ligand is FasL.

89. The vector of claim 86, wherein the conditional promoter is a tissue-specific promoter.

90. The vector of claim 89, wherein the tissue-specific promoter is selected from the group consisting of a prostate-specific promoter, a breast-specific promoter, a pancreas-specific promoter, a colon-specific promoter, a brain-specific promoter, a kidney-specific promoter, a bladder-specific promoter, a lung-specific promoter, a liver-specific promoter, a thyroid-specific promoter, a stomach-specific promoter, an ovary-specific promoter, and a cervix-specific promoter.

91. The vector of claim 89, wherein the tissue-specific promoter is a prostate-specific promoter.

92. The vector of claim 91, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB promoters.

93. The vector of claim 86, wherein the conditional promoter is an inducible promoter.

94. The vector of claim 93, wherein the inducible promoter is a promoter inducible by tetracycline or doxycycline.

95. The vector of claim 93, wherein the inducible promoter is a promoter inducible by steroid.

96. The method of claim 95, wherein the steroid is selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone.

97. An adenoviral expression vector comprising:

a tetracycline-responsive element;

a polynucleotide sequence encoding a transactivator protein which is capable of binding to the tetracycline-responsive element; and

a polynucleotide sequence encoding a target protein whose expression is regulated by the binding of the transactivator protein to the tetracycline-responsive element.

98. The vector of claim 97, wherein the tetracycline-responsive element and the polynucleotide sequence encoding the transactivator protein are positioned at opposite ends of the adenoviral vector.

99. The vector of claim 98, wherein the tetracycline-responsive element is positioned in the E4 region of the adenoviral vector and the polynucleotide sequence encoding the transactivator protein is positioned in the E1 of the adenoviral vector.

100. The vector of claim 97, wherein the adenoviral vector does not include the E3 region of adenovirus.

101. The vector of claim 97, wherein the adenoviral vector does not include the E4 region of adenovirus except for the Orf6 of the E4 region.

102. The vector of claim 97, wherein the expression of the target protein is repressed in the presence of tetracycline or doxycycline.

103. The vector of claim 97, wherein expression of the target protein is activated in the presence of doxycycline.

104. The vector of claim 97, wherein the target protein is a Fas ligand.

105. The vector of claim 97, wherein the viral expression vector further comprises a polynucleotide sequence encoding a reporter protein.

106. The vector of claim 105, wherein the reporter protein and the target protein are encoded as a fusion protein.

107. The vector of claim 105, wherein the reporter protein is a green fluorescent protein.

108. The vector of claim 97, wherein the adenoviral expression vector further comprises a polynucleotide sequence encoding a regulatory protein.

109. The vector of claim 108, wherein the regulatory protein and the target protein are encoded as a fusion protein.

110. The vector of claim 109, wherein the regulatory protein in the fusion protein is a protein that causes tissue-specific localization of the target protein.

111. An adenoviral vector that is pAd_{TET}.

112. An adenoviral vector that is $Ad/FasL\text{-}GFP_{TET}$.

Support for independent claim 1 appears on page 9, lines 4-11.

REMARKS

In light of the amendments set forth above, Applicants earnestly believe that they are entitled to a letters patent, and respectfully solicit the Examiner to expedite prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

Respectfully submitted,

Date: July 14, 2000

By: Shirley Chen

Shirley Chen, Ph.D.
Registration No. 44,608

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650 Page Mill Road
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(650) 493-9300

Attorney Docket No. 22488-708

IN THE INTERNATIONAL BUREAU
OF THE WORLD INTELLECTUAL PROPERTY ORGANIZATION

In re Patent Application of	PATENT APPLICATION
Medical University of South Carolina Foundation for Research Development	
Application No.: PCT/US99/26221	
Filed: 05 November, 1999	
Title: A Method for Treating Tumors Using Fas-Induced Apoptosis	

AMENDMENT OF THE CLAIMS UNDER ARTICLE 19

The International Bureau
World Intellectual Property Organization
34, chemin des Colombettes
1211 Geneva 20
SWITZERLAND

Dear Sir or Madam:

Responsive to the *Notification of Transmittal of the International Search Report or the Declaration* mailed, 4 May 2000, the Applicant files this *Amendment of the Claims Under Article 19* and respectfully requests inclusion of the following amendment under Article 19 in the published PCT patent application.

AMENDMENTS

Please cancel claims 1-46.

Please add the following new claims.

1. A method for inducing death in cells that express an apoptosis-mediating receptor, the method comprising:

introducing an expression vector into a group of cells comprising cells that express an apoptosis-mediating receptor, the expression vector comprising a polynucleotide sequence encoding an apoptosis-signaling ligand whose expression is regulated by a conditional promoter in the vector, the cells into which the expression vector is introduced expressing the apoptosis-signaling ligand when conditions are suitable to activate the conditional promoter, the expressed apoptosis-signaling ligand inducing cell death in those cells which express the apoptosis-mediating receptor through interaction between the apoptosis-signaling ligand and the apoptosis-mediating receptor.

2. The method of claim 1, wherein the apoptosis-mediating receptor is a membrane-bound receptor.

3. The method of claim 2, wherein the membrane-bound receptor is Fas.

4. The method of claim 3, wherein the apoptosis-signaling ligand is capable of binding to Fas.

5. The method of claim 4, wherein the apoptosis-signaling ligand is an antibody that is capable of binding to Fas and signals Fas-mediated apoptosis in cells expressing Fas.

6. The method of claim 4, wherein the apoptosis-signaling ligand is a membrane protein.

7. The method of claim 6, wherein the membrane protein is FasL.

8. The method of claim 3, wherein the group of cells into which the expression vector is introduced comprises a mixture of cells which express Fas and cells which do not express Fas.

9. The method of claim 3, wherein the expression vector is introduced into cells which do not express Fas.

10. The method of claim 3, wherein the expression vector is introduced into cells which do express Fas.

11. The method of claim 3, wherein the expression vector is introduced into cells which do not express Fas and cells which do express Fas.

12. The method of claim 1, wherein the group of cells are contained in a solid tumor.

13. The method of claim 12, wherein the solid tumor is selected from the group consisting of breast, prostate, brain, bladder, pancreas, rectum, parathyroid, thyroid, adrenal, head and neck, colon, stomach, bronchi and kidney tumors.

14. The method of claim 1, wherein introducing an expression vector into the group of cells is performed parenterally, intraperitoneally, intravenously, intraartierally, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery by catheter or stent, subcutaneously, intraadiposally, intraarticularly, intrathecally, or in a slow release dosage form.

15. The method of claim 1, wherein introducing the expression vector is performed by direct injection of the expression vector among the group of cells.

16. The method of claim 1, wherein the expression vector is a plasmid.

17. The method of claim 1, wherein the expression vector is a viral vector.

18. The method of claim 17, wherein the viral vector is selected from the group consisting of adenovirus, adeno-associated virus, vaccinia, retrovirus, and herpes simplex virus vectors.

19. The method of claim 17, wherein the expression vector is an adenoviral vector.

20. The method of claim 1, wherein the conditional promoter is a tissue-specific promoter.

21. The method of claim 20, wherein the tissue-specific promoter is selected from the group consisting of a prostate-specific promoter, a breast-specific promoter, a pancreas-specific promoter, a colon-specific promoter, a brain-specific promoter, a kidney-specific promoter, a bladder-specific promoter, a lung-specific promoter, a liver-specific promoter, a thyroid-specific promoter, a stomach-specific promoter, an ovary-specific promoter, and a cervix-specific promoter.

22. The method of claim 1, wherein the group of cells are prostate cancer cells and the conditional promoter of the expression vector is a prostate-specific promoter.

23. The method of claim 22, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB promoters.

24. The method of claim 1, wherein the conditional promoter is an inducible promoter.

25. The method of claim 24, wherein the inducible promoter is a promoter inducible by tetracycline or doxycycline.

26. The method of claim 24, wherein the inducible promoter is a promoter inducible by steroid.

27. The method of claim 26, wherein the steroid is selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone.

28. The method of claim 1, the method further comprising creating the conditions suitable to activate the conditional promoter.

29. The method of claim 28, wherein creating the conditions suitable to activate the conditional promoter comprises delivering to the group of cells tetracycline or deoxycycline.

30. The method of claim 28, wherein creating the conditions suitable to activate the conditional promoter comprises delivering to the group of cells a steroid selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone .

31. The method of claim 1, wherein the expression vector further comprises a reporter gene.

32. The method of claim 31, wherein the expression vector expresses the reporter gene as a fusion protein with the apoptosis-signaling ligand.

33. The method of claim 32, wherein the reporter gene encodes green fluorescent protein.

34. The method of claim 1, wherein the expression vector further comprises a polynucleotide sequence encoding a regulatory protein.

35. The method of claim 34, wherein the expression vector expresses the regulatory protein as a fusion protein with the apoptosis-signaling ligand.

36. The method of claim 35, wherein the regulatory protein in the fusion protein is a protein that causes tissue-specific localization of the apoptosis-signaling ligand.

37. The method of claim 1, wherein the method is performed *ex vivo* where the group of cells into which the expression vector is introduced are contained in a sample taken from a patient having cancer.

38. The method of claim 1, wherein the method is performed *in vitro* where the group of cells into which the expression vector is introduced are contained in a cell culture.

39. The method of claim 1, wherein the apoptosis-signaling ligand is selected from the group consisting of Bax, Bad, Bak, and Bik.

40. An adenoviral expression vector comprising:

a conditional promoter, and

a polynucleotide sequence encoding a membrane-bound ligand whose expression is regulated by the conditional promoter in the vector, the ligand signaling apoptosis in cells that express an apoptosis-mediating receptor.

41. The vector of claim 40, wherein the membrane-bound ligand is capable of binding to Fas.

42. The method of claim 41, wherein the membrane-bound ligand is FasL.

43. The vector of claim 40, wherein the conditional promoter is a tissue-specific promoter.

44. The vector of claim 43, wherein the tissue-specific promoter is selected from the group consisting of a prostate-specific promoter, a breast-specific promoter, a pancreas-specific promoter, a colon-specific promoter, a brain-specific promoter, a kidney-specific promoter, a bladder-specific promoter, a lung-specific promoter, a liver-specific promoter, a thyroid-specific promoter, a stomach-specific promoter, an ovary-specific promoter, and a cervix-specific promoter.

45. The vector of claim 43, wherein the tissue-specific promoter is a prostate-specific promoter.

46. The vector of claim 45, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB promoters.

47. The vector of claim 40, wherein the conditional promoter is an inducible promoter.

48. The vector of claim 47, wherein the inducible promoter is a promoter inducible by tetracycline or doxycycline.

49. The vector of claim 47, wherein the inducible promoter is a promoter inducible by steroid.

50. The method of claim 49, wherein the steroid is selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone.

51. An adenoviral expression vector comprising:

a tetracycline-responsive element;

a polynucleotide sequence encoding a transactivator protein which is capable of binding to the tetracycline-responsive element; and

a polynucleotide sequence encoding a target protein whose expression is regulated by the binding of the transactivator protein to the tetracycline-responsive element.

52. The vector of claim 51, wherein the tetracycline-responsive element and the polynucleotide sequence encoding the transactivator protein are positioned at opposite ends of the adenoviral vector.

53. The vector of claim 52, wherein the tetracycline-responsive element is positioned in the E4 region of the adenoviral vector and the polynucleotide sequence encoding the transactivator protein is positioned in the E1 of the adenoviral vector.

54. The vector of claim 51, wherein the adenoviral vector does not include the E3 region of adenovirus.

55. The vector of claim 51, wherein the adenoviral vector does not include the E4 region of adenovirus except for the Orf6 of the E4 region.

56. The vector of claim 51, wherein the expression of the target protein is repressed in the presence of tetracycline or doxycycline.

57. The vector of claim 51, wherein expression of the target protein is activated in the presence of doxycycline.

58. The vector of claim 51, wherein the target protein is a Fas ligand.

59. The vector of claim 51, wherein the viral expression vector further comprises a polynucleotide sequence encoding a reporter protein.

60. The vector of claim 59, wherein the reporter protein and the target protein are encoded as a fusion protein.

61. The vector of claim 59, wherein the reporter protein is a green fluorescent protein.

62. The vector of claim 51, wherein the adenoviral expression vector further comprises a polynucleotide sequence encoding a regulatory protein.

63. The vector of claim 62, wherein the regulatory protein and the target protein are encoded as a fusion protein.

64. The vector of claim 63, wherein the regulatory protein in the fusion protein is a protein that causes tissue-specific localization of the target protein.

65. An adenoviral vector that is pAd_{TET}.

66. An adenoviral vector that is $Ad/FasL\text{-}GFP_{TET}$.

Support for independent claim 1 appears on page 9, lines 4-11.

Enclosed are substitute pages 44 through 48 of the claims.

Respectfully submitted,

Dated: June 30, 2000

By: ______________________

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What is claimed is:

1. A method for inducing death in cells that express an apoptosis-mediating receptor, the method comprising:

introducing an expression vector into a group of cells comprising cells that express an apoptosis-mediating receptor, the expression vector comprising a polynucleotide sequence encoding an apoptosis-signaling ligand whose expression is regulated by a conditional promoter in the vector, the cells into which the expression vector is introduced expressing the apoptosis-signaling ligand when conditions are suitable to activate the conditional promoter, the expressed apoptosis-signaling ligand inducing cell death in those cells which express the apoptosis-mediating receptor through interaction between the apoptosis-signaling ligand and the apoptosis-mediating receptor.

2. The method of claim 1, wherein the apoptosis-mediating receptor is a membrane-bound receptor.

3. The method of claim 2, wherein the membrane-bound receptor is Fas.

4. The method of claim 3, wherein the apoptosis-signaling ligand is capable of binding to Fas.

5. The method of claim 4, wherein the apoptosis-signaling ligand is an antibody that is capable of binding to Fas and signals Fas-mediated apoptosis in cells expressing Fas.

6. The method of claim 4, wherein the apoptosis-signaling ligand is a membrane protein.

7. The method of claim 6, wherein the membrane protein is FasL.

8. The method of claim 3, wherein the group of cells into which the expression vector is introduced comprises a mixture of cells which express Fas and cells which do not express Fas.

9. The method of claim 3, wherein the expression vector is introduced into cells which do not express Fas.

10. The method of claim 3, wherein the expression vector is introduced into cells which do express Fas.

11. The method of claim 3, wherein the expression vector is introduced into cells which do not express Fas and cells which do express Fas.

12. The method of claim 1, wherein the group of cells are contained in a solid tumor.

13. The method of claim 12, wherein the solid tumor is selected from the group consisting of breast, prostate, brain, bladder, pancreas, rectum, parathyroid, thyroid, adrenal, head and neck, colon, stomach, bronchi and kidney tumors.

14. The method of claim 1, wherein introducing an expression vector into the group of cells is performed parenterally, intraperitoneally, intravenously, intraartierally, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery by catheter or stent, subcutaneously, intraadiposally, intraarticularly, intrathecally, or in a slow release dosage form.

15. The method of claim 1, wherein introducing the expression vector is performed by direct injection of the expression vector among the group of cells.

16. The method of claim 1, wherein the expression vector is a plasmid.

17. The method of claim 1, wherein the expression vector is a viral vector.

18. The method of claim 17, wherein the viral vector is selected from the group consisting of adenovirus, adeno-associated virus, vaccinia, retrovirus, and herpes simplex virus vectors.

19. The method of claim 17, wherein the expression vector is an adenoviral vector.

20. The method of claim 1, wherein the conditional promoter is a tissue-specific promoter.

21. The method of claim 20, wherein the tissue-specific promoter is selected from the group consisting of a prostate-specific promoter, a breast-specific promoter, a pancreas-specific promoter, a colon-specific promoter, a brain-specific promoter, a kidney-specific promoter, a bladder-specific promoter, a lung-specific promoter, a liver-specific promoter, a thyroid-specific promoter, a stomach-specific promoter, an ovary-specific promoter, and a cervix-specific promoter.

22. The method of claim 1, wherein the group of cells are prostate cancer cells and the conditional promoter of the expression vector is a prostate-specific promoter.

23. The method of claim 22, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB promoters.

24. The method of claim 1, wherein the conditional promoter is an inducible promoter.

25. The method of claim 24, wherein the inducible promoter is a promoter inducible by tetracycline or doxycycline.

26. The method of claim 24, wherein the inducible promoter is a promoter inducible by steroid.

27. The method of claim 26, wherein the steroid is selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone.

28. The method of claim 1, the method further comprising creating the conditions suitable to activate the conditional promoter.

29. The method of claim 28, wherein creating the conditions suitable to activate the conditional promoter comprises delivering to the group of cells tetracycline or deoxycycline.

30. The method of claim 28, wherein creating the conditions suitable to activate the conditional promoter comprises delivering to the group of cells a steroid selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone .

31. The method of claim 1, wherein the expression vector further comprises a reporter gene.

32. The method of claim 31, wherein the expression vector expresses the reporter gene as a fusion protein with the apoptosis-signaling ligand.

33. The method of claim 32, wherein the reporter gene encodes green fluorescent protein.

34. The method of claim 1, wherein the expression vector further comprises a polynucleotide sequence encoding a regulatory protein.

35. The method of claim 34, wherein the expression vector expresses the regulatory protein as a fusion protein with the apoptosis-signaling ligand.

36. The method of claim 35, wherein the regulatory protein in the fusion protein is a protein that causes tissue-specific localization of the apoptosis-signaling ligand.

37. The method of claim 1, wherein the method is performed *ex vivo* where the group of cells into which the expression vector is introduced are contained in a sample taken from a patient having cancer.

38. The method of claim 1, wherein the method is performed *in vitro* where the group of cells into which the expression vector is introduced are contained in a cell culture.

39. The method of claim 1, wherein the apoptosis-signaling ligand is selected from the group consisting of Bax, Bad, Bak, and Bik.

40. An adenoviral expression vector comprising:

a conditional promoter, and

a polynucleotide sequence encoding a membrane-bound ligand whose expression is regulated by the conditional promoter in the vector, the ligand signaling apoptosis in cells that express an apoptosis-mediating receptor.

41. The vector of claim 40, wherein the membrane-bound ligand is capable of binding to Fas.

42. The method of claim 41, wherein the membrane-bound ligand is FasL.

43. The vector of claim 40, wherein the conditional promoter is a tissue-specific promoter.

44. The vector of claim 43, wherein the tissue-specific promoter is selected from the group consisting of a prostate-specific promoter, a breast-specific promoter, a pancreas-specific promoter, a colon-specific promoter, a brain-specific promoter, a kidney-specific promoter, a bladder-specific promoter, a lung-specific promoter, a liver-specific promoter, a thyroid-specific promoter, a stomach-specific promoter, an ovary-specific promoter, and a cervix-specific promoter.

45. The vector of claim 43, wherein the tissue-specific promoter is a prostate-specific promoter.

46. The vector of claim 45, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB promoters.

47. The vector of claim 40, wherein the conditional promoter is an inducible promoter.

48. The vector of claim 47, wherein the inducible promoter is a promoter inducible by tetracycline or doxycycline.

49. The vector of claim 47, wherein the inducible promoter is a promoter inducible by steroid.

50. The method of claim 49, wherein the steroid is selected from the group consisting of glucocorticoid, estrogen, androgen, and progestrone.

51. An adenoviral expression vector comprising:

a tetracycline-responsive element;

a polynucleotide sequence encoding a transactivator protein which is capable of binding to the tetracycline-responsive element; and

a polynucleotide sequence encoding a target protein whose expression is regulated by the binding of the transactivator protein to the tetracycline-responsive element.

52. The vector of claim 51, wherein the tetracycline-responsive element and the polynucleotide sequence encoding the transactivator protein are positioned at opposite ends of the adenoviral vector.

53. The vector of claim 52, wherein the tetracycline-responsive element is positioned in the E4 region of the adenoviral vector and the polynucleotide sequence encoding the transactivator protein is positioned in the E1 of the adenoviral vector.

54. The vector of claim 51, wherein the adenoviral vector does not include the E3 region of adenovirus.

55. The vector of claim 51, wherein the adenoviral vector does not include the E4 region of adenovirus except for the Orf6 of the E4 region.

56. The vector of claim 51, wherein the expression of the target protein is repressed in the presence of tetracycline or doxycycline.

57. The vector of claim 51, wherein expression of the target protein is activated in the presence of doxycycline.

58. The vector of claim 51, wherein the target protein is a Fas ligand.

59. The vector of claim 51, wherein the viral expression vector further comprises a polynucleotide sequence encoding a reporter protein.

60. The vector of claim 59, wherein the reporter protein and the target protein are encoded as a fusion protein.

61. The vector of claim 59, wherein the reporter protein is a green fluorescent protein.

62. The vector of claim 51, wherein the adenoviral expression vector further comprises a polynucleotide sequence encoding a regulatory protein.

63. The vector of claim 62, wherein the regulatory protein and the target protein are encoded as a fusion protein.

64. The vector of claim 63, wherein the regulatory protein in the fusion protein is a protein that causes tissue-specific localization of the target protein.

65. An adenoviral vector that is pAd_{TET}.

66. An adenoviral vector that is $Ad/FasL\text{-}GFP_{TET}$.

A METHOD OF TREATING TUMORS USING FAS-INDUCED APOPTOSIS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention provides a method of killing a Fas^+ tumor cell comprising introducing into a second tumor cell a nucleic acid encoding a Fas ligand (FasL), whereby the second tumor cell expresses the nucleic acid thereby producing FasL, and whereby interaction of the Fas^+ tumor cell with the second tumor cell expressing FasL causes the Fas^+ tumor cell to undergo apoptosis, thereby killing the Fas^+ tumor cell.

BACKGROUND ART

Currently, a major treatment for cancerous tumors is surgical removal of the affected areas of the tissue, organ, or gland. However, high recurrence rates are a major obstacle to the complete eradication of cancerous cells. One reason for frequent tumor recurrence is that during the development of the primary cancer, complete removal of all the cancer cells by surgical procedures is extremely difficult. The remaining cancer cells often remain quiescent for extended periods of time, which is termed tumor dormancy (*Meltzer et al.* 1990. ADormancy and breast cancer.@ J. Surg. Oncol. 43:181-188). Once the primary tissue is surgically removed, the surgical injury can stimulate rapid tissue and blood vessel regeneration at the wound. These regeneration processes send out positive signals to the surrounding tissue, for example by tissue and vessel growth factors. These factors and the rapid proliferative environment induce the transition of the remaining tumor cells from dormancy to rapid proliferation, and thereby cause reoccurrence of the cancer.

Two basic features are shared by all cancer cells: the uncontrolled cell cycling; and the inability to enter the pathway of programmed cell death, apoptosis. Apoptosis is an intrinsic property of all normal cells. The apoptotic process has important roles in regulating the development of tissues, the sizes and shapes of organs, and the life span of cells. Apoptosis acts as a safeguard to prevent overgrowth of cells and tissues. Fas-mediated apoptosis is the best-studied pathway of programmed cell death. Fas (APO-1, CD95), or the Fas ligand receptor, is a 45 kDa type I membrane protein and belongs to the TNF/nerve growth factor receptor superfamily Bajorath, J. and A. Aruffo. 1997. Prediction of the three-dimensional structure of the human Fas receptor by comparative molecular modeling. J Comput Aided Mol Des 11:3-8 and Watanabe-Fukunaga, R., C. I. Brannan, N. Itoh, S. Yonehara, N. G. Copeland, N. A. Jenkins and S. Nagata. 1992. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 148:1274-9. The ligand of Fas, FasL, is a 40-kDa type II membrane protein belonging to the tumor necrosis factor family Takahashi, T., M. Tanaka, J. Inazawa, T. Abe, T. Suda and S. Nagata. 1994. Human Fas ligand: gene structure, chromosomal location and species specificity. Int Immunol **6**:1567-74. Binding of FasL (and certain anti-Fas antibodies) to Fas causes receptor oligomerization and sends a signal through a caspase pathway, resulting in rapid death of receptor-bearing cells through apoptosis. (Larsen, C. P., D. Z. Alexander, R. Hendrix, S. C. Ritchie and T. C. Pearson. 1995. Fas-mediated cytotoxicity. An immunoeffector or immunoregulatory pathway in T cell-mediated immune responses? Transplantation 60:221-4; Longthorne, V. L. and G. T. Williams. 1997.) Caspase activity is required for commitment to Fas-mediated apoptosis. (Embo J 16:3805-12; Nagata, S. and P. Golstein. 1995. The Fas death factor. Science 267:1449-56; Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice [published erratum appears in Nature 1993 Oct 7;365(6446):568]. Nature 364:806-9.) Fas is expressed in almost all cell types. When Fas binds to FasL, it activates the genetically programmed cell death through a cascade expression of interleukin-coupled enzymes

(ICE) or caspases (*Chandler et al.* 1998 " Different subcellular distribution of caspase-3 and caspase-7 following Fas-induced apoptosis in mouse liver" J. Biol. Chem. 273:10815-10818; *Jones et al.* 1998 "Fas-mediated apoptosis in mouse hepatocytes involves the processing and activation of caspases" Hepatology 27:1632-1642).

Since both ligand and receptor are membrane proteins, Fas-induced apoptosis is normally mediated through cell-cell contact. However, a soluble form of FasL is also produced by some cells and has been shown to have a somewhat altered activity, depending on the target cell Tanaka, M., T. Itai, M. Adachi and S. Nagata. 1998. Downregulation of Fas ligand by shedding [see comments]. Nat Med 4:31-6; Tanaka, M., T. Suda, T. Takahashi and S. Nagata. 1995. Expression of the functional soluble form of human fas ligand in activated lymphocytes. Embo J 14:1129-35.

This invention provides a novel strategy to destroy the primary tumor and, at the same time, prevent the reoccurrence of the cancer by activating cancer cell apoptosis, such as via vector-mediated gene transfer of a Fas ligand to a cell. In this method, the cell now expressing the Fas ligand induces Fas^+ tumor cells to undergo apoptosis and die. The vector can be injected into the tumor with a syringe or a micropump, thus eliminating the need for conventional surgery to remove the tumor. In the present invention, cancer cell death is induced in several ways: 1) FasL binds to Fas receptors on adjacent tumor cells and induces their apoptosis; 2) Fas L induces apoptosis of endothelial cells and destroys the blood vessels supplying the tumor; 3) expression of FasL on tumor cells induces apoptosis of surrounding tissues and deprives tumor cells of any nursery support; and 4) apoptosis prevents the release of positive factors that may reactivate quiescent tumor cells responsible for reoccurring cancers.

A major advantage of this approach is that the Fas-FasL interaction is the major signaling event that activates several apoptosis pathways, both p53-dependent and independent pathways (*Callera et al.* 1998 "Fas-mediated apoptosis with normal

expression of bcl-2 and p53 in lymphocytes from aplastic anemia" Br. J. Haematol. 100:698-703). Thus, apoptosis signaling is amplified by more than one cascade of enzyme expressions, and the apoptosis does not depend on p53 or other cell-cycle checkpoint proteins. For example, although gene therapy with the p53 gene has shown great promise in treating cancers (*Boulikas* 1997 "Gene therapy of prostate cancer: p53, suicidal genes, and other targets." Anticancer Res. 17:1471-1505), p53 gene therapy will only work in about 50-60% of the tumor cells that have a p53 mutation (*Iwaya et al.* 1997 AHistologic grade and p53 immunoreaction as indicators of early recurrence of node-negative breast cancer. Jpn J Clin Oncol 27:6-12). Another advantage is that FasL is generally a membrane-bound signaling protein rather than an intracellular protein, such as p53 and caspases. FasL expression on the cell surface transmits the apoptotic signal to surrounding cancer cells by a strong Abystander effect@, and does not require delivering the therapeutic gene into all cancer cells. Therefore, the present invention fufills the need for a nonsurgical method of cancer treatment that provides significant improvement over current gene therapy methods, avoids the use of toxic drugs and helps prevent tumor recurrence.

The present invention provides a method for the delivery of FasL for the purpose of destroying tumor cells by providing a means for delivering FasL to a wide range of cell types both *in vitro* and *in vivo*, a means for tight regulation of FasL expression, and a means for easily and reliably quantitating the levels and cellular localization of exogenous FasL.

SUMMARY OF THE INVENTION

The present invention provides a method of killing a Fas^+ tumor cell comprising introducing into a second tumor cell a nucleic acid encoding a Fas ligand (FasL), whereby the second tumor cell expresses the nucleic acid thereby producing FasL, and whereby interaction of the Fas^+ tumor cell with the second tumor cell expressing FasL

causes the Fas^+ tumor cell to undergo apoptosis, thereby killing the Fas^+ tumor cell.

In another embodiment, the invention provides a method of killing a Fas^+ tumor cell comprising introducing into a second tumor cell the vector $Ad/FasL\text{-}GFP_{TET}$, whereby the second tumor cell expresses FasL, and whereby interaction of the Fas^+ tumor cell with the second tumor cell expressing FasL causes the Fas^+ tumor cell to undergo apoptosis, thereby killing the Fas^+ tumor cell. In yet another embodiment, the invention provides the vector $Ad/FasL\text{-}GFP_{TET}$.

The invention also provides a regulatable expression vector comprising a nucleic acid encoding a transactivator protein that binds to a tet-responsive transactivating expression element, and a regulatory element comprising a tet-responsive transactivating expression element, wherein a nucleic acid encoding a protein to be expressed may be inserted downstream of the regulatory region. One such vector provided by the invention is pAd_{TET}.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A, 1B, and 1 C schematically show the pLAd-C.tTA vector, the pRAd.T.GFsL vector, and the $rAd/FasL\text{-}GFP_{TET}$ vector, respectively. In Figure 1A, the pLAd-C.tTA vector is shown. This plasmid contains the leftmost 450 bp of Ad5 genome, followed by a strong CMVie enhancer/promoter and a tTA gene from pUHD15-1 inserted into the MCS. Adapter contains restriction sites Xba1, Avr2 and Spe1, all of which generate cohesive ends compatible with Xba1. After assembly into rAd vectors, E1A poly A is utilized for efficient tTA expression. A similar strategy was used to construct pLAd vectors containing other transgenes. In Figure 1B, the pRAd.T.GFsL vector is shown. This plasmid contains Ad5 (sub360) sequences from the unique EcoR1 site (27333 bp) to the right ITR (35935 bp), with E3 and E4 deletions (the Orf6 of E4 is retained). The diagram shows the structure of the regulatable FasL-

GFP expression cassette, consisting of the TRE promoter, FasL-GFP fusion protein and bovine groth hormone (BGH) poly A. This cassette was inserted into a MCS at 35810 bp. *In vitro* assembly of the rAd/FasL-GFP$_{TET}$ vector is shown in Figure 1C. The region of the junction between the GFP and FasL reading frames is expanded. Other rAd vectors were generated using a similar strategy.

Figure 2 is a graph showing a comparison of titers of rAd vectors with FasL activity in 293 and 293CrmA cells. 12 well plates were seeded with 10^4 293 or 293CrmA cells and infected with r-Ad/FasL, rAdFasL-GFP$_{TET}$, or rAd/LacZ at MOI of 5 one day later. 48 hours post-transduction, cells were collected and lysed. Lysates were titrated and PFU/ml determined on 293CrmA cells. Results represent means and average errors of 2 sets of independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included therein.

Before the present methods are disclosed and described, it is to be understood that this invention is not limited to specific compounds and methods, as such may of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, a cell can mean a single cell or more than one cell.

The present invention provides a method of treating tumor containing a Fas$^+$

tumor cell comprising introducing into a second tumor cell a nucleic acid encoding a Fas ligand (FasL), whereby the second tumor cell expresses the nucleic acid thereby producing FasL, and whereby interaction of the Fas^+ tumor cell with the second tumor cell expressing FasL causes the Fas^+ tumor cell to undergo apoptosis, thereby treating the tumor containing the Fas^+ tumor cell.

One skilled in the art will appreciate that there are numerous techniques available by which one can obtain a nucleic acid sequence encoding a Fas ligand, and optionally, additional sequences such as one or more regulatory sequence. One method of obtaining the nucleic acid is by constructing the nucleic acid by synthesizing a recombinant DNA molecule. For example, oligonucleotide synthesis procedures are routine in the art and oligonucleotides coding for a particular protein or regulatory region are readily obtainable through automated DNA synthesis. A nucleic acid for one strand of a double-stranded molecule can be synthesized and hybridized to its complementary strand. One can design these oligonucleotides such that the resulting double-stranded molecule has either internal restriction sites or appropriate 5' or 3' overhangs at the termini for cloning into an appropriate vector. Double-stranded molecules coding for relatively large proteins or regulatory regions can be synthesized by first constructing several different double-stranded molecules that code for particular regions of the protein or regulatory region, followed by ligating these DNA molecules together. For example, *Cunningham, et al.*, AReceptor and Antibody Epitopes in Human Growth Hormone Identified by Homolog-Scanning Mutagenesis,@ *Science*, Vol. 243, pp. 1330-1336 (1989), have constructed a synthetic gene encoding the human growth hormone gene by first constructing overlapping and complementary synthetic oligonucleotides and ligating these fragments together. See also, *Ferretti et al.*, Proc. Nat. Acad. Sci. 82:599-603 (1986), wherein synthesis of a 1057 base pair synthetic bovine rhodopsin gene from synthetic oligonucleotides is disclosed. Once the appropriate DNA molecule is synthesized, this DNA can be cloned downstream of an appropriate promoter. Techniques such as this are routine in the art and are well

documented.

An example of another method of obtaining a nucleic acid encoding a Fas ligand is to isolate the corresponding wild-type nucleic acid from the organism in which it is found and clone it in an appropriate vector. For example, a DNA or cDNA library can be constructed and screened for the presence of the nucleic acid of interest. Methods of constructing and screening such libraries are well known in the art and kits for performing the construction and screening steps are commercially available (for example, Stratagene Cloning Systems, La Jolla, CA). Once isolated, the nucleic acid can be directly cloned into an appropriate vector, or if necessary, be modified to facilitate the subsequent cloning steps. Such modification steps are routine, an example of which is the addition of oligonucleotide linkers which contain restriction sites to the termini of the nucleic acid. General methods are set forth in *Sambrook et al.*, AMolecular Cloning, a Laboratory Manual,@ Cold Spring Harbor Laboratory Press (1989). Once isolated, one can alter selected codons using standard laboratory techniques, PCR for example.

Yet another example of a method of obtaining a nucleic acid encoding a Fas ligand is to amplify the corresponding wild-type nucleic acid from the nucleic acids found within a host organism containing the wild-type nucleic acid and clone the amplified nucleic acid in an appropriate vector. One skilled in the art will appreciate that the amplification step may be combined with a mutation step, using primers not completely homologous to the target nucleic acid for example, to simultaneously amplify the nucleic acid and alter specific positions of the nucleic acid.

The nucleic acid encoding a FasL can be a DNA, an RNA, or any combination thereof, whether containing only those bases typically found, or containing modified bases. These modified nucleotides are well known in the art and include, but are not limited to, thio-modified deoxynucleotide triphosphates and borano-modified

deoxynucleotide triphosphates (Eckstein and Gish, *Trends in Biochem. Sci.*, 14:97-100 (1989) and Porter *Nucleic Acids Research*, 25:1611-1617 (1997)).

Alternatively, the nucleic acid can encode another type of signaling ligand and/or receptor such that when that ligand and/or receptor is introduced into a cell, and whereby the cell expresses the nucleic acid thereby producing the ligand and/or receptor, the interaction of the ligand and/or the receptor causes a tumor cell to undergo apoptosis, thereby treating the tumor cell. An example of other signal molecules that can be used in the methods of the present invention includes, but is not limited to, Bax, Bad, Bak, and Bik. (*Adams et al.* "Control of cell death" WEHI Annual Report 1996/1997).

In another embodiment of the present invention, the nucleic acid encoding the Fas ligand can also encode another protein such as a regulatory protein, which may be used to regulate the expression of the Fas ligand. For example, the regulatory protein can cause the tissue-specific localization of the Fas ligand on the cell membrane, or alternatively cause the premature turn-over of the Fas ligand in non-target cells, or regulate the expression of the FasL via regulation of transcription and/or translation.

The regulatory protein can also be encoded by another nucleic acid that is delivered to the cell, either concurrently or consecutively with the nucleic acid encoding the protein to be expressed. In this embodiment, the two nucleic acids can have different sequences, such as different promoters, such that they can be independently regulated, such as by the administration of a drug that selectively regulates the expression of one or both of the promoters, such as by the use of a steroid hormone, e.g. a glucocorticoid hormone that can regulate a promoter that is inducible by that hormone.

The nucleic acid encoding a Fas ligand can also comprise a fusion protein. One skilled in the art will recognize that fusion proteins are routinely used for such purposes

as localization of the protein, activation or deactivation of the protein, monitoring the location of the protein, isolation of the protein, and quantitating the amount of the protein. In one embodiment, the fusion protein comprises a Fas ligand and a green fluorescent protein. Other examples of fusion proteins that comprise the Fas ligand include the GFP gene, the CAT gene, the neo gene, the hygromycin gene, and so forth. Anexample of a FasL-GFP fusion protein-expressing construct is shown in Figure 1 and is further described herein.

The nucleic acid encoding a Fas ligand can also contain a sequence that is capable of regulating the expression of the Fas ligand. For example, the nucleic acid can contain a glucocorticoid regulatory element (GRE) such that glucocorticoid hormones can be used to regulate the expression of the Fas ligand. Another example of a regulatory sequence that can regulate the expression of an adjacent gene is by cloning an RNA aptamer, such as H10 and H19, into the promoter region whereby administration of a drug such as Hoechst dye 33258 can block expression of the gene in vivo. (*Werstuck et al.* "Controlling gene expression in living cells through small molecule-RNA interactions" Science 282:296-298 (1998)). In other embodiments of the present invention, the regulatory sequence comprises the Tet-operon or the lac operon, or any other operon that can function as a regulatory sequence in a eukaryotic cell.

In a preferred embodiment, expression of FasL protein is under the control of tetracycline-regulated gene expression system, wherein expression of FasL is controlled by a tet-responsive element, wherein FasL expression requires the interaction of the tet-responsive element and a tet transactivator. In a more preferred embodiment, tight control of FasL expression is achieved using an Ad vector in which the tet-responsive element and the transactivator element are built into the opposite ends of the same vector to avoid enhancer interference. Expression can be conveniently regulated by tetracycline or any derivative thereof, which includes, but is not limited to, doxycycline,

in a dose-dependent manner. The vector efficiently delivers FasL-GFP gene to cells *in vitro*, and the expression level of the fusion protein may be modulated by the concentration of doxycycline in culture media. An example of such a regulatory system is particularly described herein.

The methods described herein comprise introducing into a cell a nucleic acid encoding a Fas ligand. One skilled in the art will recognize that this aspect of the methods can comprise either a stable or a transient introduction of the nucleic acid construct into the cell. Additionally, the stably or the transiently introduced nucleic acid may or may not become integrated into the genome of the host. One skilled in the art will also recognize that the precise procedure for introducing the nucleic acid into the cell may, of course, vary and may depend on the specific type or identity of the cell. Examples of methods for introducing a nucleic acid into a cell include, but are not limited to electroporation, cell fusion, DEAE-dextran mediated transfection, calcium phosphate-mediated transfection, infection with a viral vector, microinjection, lipofectin-mediated transfection, liposome delivery, and particle bombardment techniques, including various procedures for "naked DNA" delivery. The cell into which a nucleic acid encoding FasL is introduced can be a Fas-expressing cell or a cell not expressing Fas.

In one embodiment of the present invention, the promoter is a tissue-specific promoter which one skilled in the art will appreciate can confer tissue-specificity to the expression of the nucleic acid encoding the FasL. For example, the tissue-specific promoter may be a prostate-specific, a breast tissue-specific, a colon tissue-specific, a brain-specific, a kidney-specific, a liver-specific, a bladder-specific, a lung-specific, a thyroid-specific a stomach-specific, a ovary-specific, or a cervix-specific promoter. Where the tissue-specific promoter is a prostate-specific promoter, the promoter includes, but is not limited to the PSA promoter, the ΔPSA promoter, the ARR2PB promoter, and the PB promoter. Where the tissue-specific promoter is a breast-specific

promoter, the promoter includes, but is not limited to MMTV and whey acidic protein promoters. Where the tissue-specific promoter is a liver-specific promoter, the promoter includes, but is not limited to the albumin and alpha fetoprotein promoters. Where the tissue-specific promoter is a brain-specific promoter, the promoter includes, but is not limited to, the JC virus early promoter, and the tyrosine hydoxylase and dopamine promoters. Where the tissue-specific promoter is a brain-specific promoter, the promoter includes, but is not limited to, the JC virus early promoter, and the tyrosine hydoxylase, dopamine hydroxylase, neuron specific enolase, and glial fibrillary acidic protein promoters. Where the tissue-specific promoter is a colon-specific promoter, the promoter includes, but is not limited to, the MUC1 promoter. Where the tissue-specific promoter is a colon-specific promoter, the promoter includes, but is not limited to, the MUC1 promoter. Of course, other tissue specific promoters will be revealed by the human genome project. These promoters will be useable as appropriate to direct tissue specific expression from the present vectors.

Futhermore, one of ordinary skill will readily know how to identify a promoter specific to a particular cell type. For example, by comparing the differential expression of genes in different tissue types, e.g., using gene chip technology, one can identify genes expressed only in one particular tissue type. These genes can then be isolated and sequenced, and their promoters may be isolated and tested in an animal model for the ability to drive tissue specific expression of a heterologous gene. Such methods are well within the ability of the one of ordinary skill in the art. An example of a method by which a tissue specific promoter may be identified may be found in Greenberg et al. (Molecular Endocrinology 8: 230-239, 1994).

The tissue-specificity can also be achieved by selecting a vector that has a high degree of tissue specificity. For example, a vector that selectively infects mucosal cells, such as those associated with colon cancer, can be chosen, and then optionally, used in combination with a specific delivery means, such as by the use of a suppository, to

selectively deliver the nucleic acid encoding FasL to those desired cells.

One skilled in the art will recognize that various vectors have more or less applicability depending on the particular host. One example of a particular technique for introducing nucleic acids into a particular host is the use of retroviral vector systems which can package a recombinant retroviral genome. (See e.g., *Pastan et al.* "A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells." Proc. Nat. Acad. Sci. 85:4486 (1988) and *Miller et al.* "Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production." Mol. Cell Biol. 6:2895 (1986)). The produced recombinant retrovirus can then be used to infect and thereby deliver to the infected cells a nucleic acid sequence encoding a Fas ligand. The exact method of introducing the nucleic acid into mammalian cells is, of course, not limited to the use of retroviral vectors. Other techniques are widely available for this procedure including the use of adenoviral vectors (*Mitani et al.* "Transduction of human bone marrow by adenoviral vector." Human Gene Therapy 5:941-948 (1994)), adenoassociated viral vectors (*Goodman et al.* "Recombinant adenoassociated virus-mediated gene transfer into hematopoietic progenitor cells." Blood 84:1492-1500 (1994)), lentiviral vectors (*Naidini et al.* "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector." Science 272:263-267 (1996)), pseudotyped retroviral vectors (*Agrawal et al.* "Cell-cycle kinetics and VSV-G pseudotyped retrovirus mediated gene transfer in blood-derived $CD34^+$ cells." Exp. Hematol. 24:738-747 (1996)), vaccinia vectors, and physical transfection techniques (*Schwarzenberger et al.* "Targeted gene transfer to human hematopoietic progenitor cell lines through the *c-kit* receptor." Blood 87:472-478 (1996)). This invention can be used in conjunction with any of these or other commonly used gene transfer methods. In a preferred embodiment of the present invention, the specific vector for delivering the nucleic acid encoding a Fas ligand comprises an adenovirus vector.

Because it is desirable to be able to regulate expression of FasL or a FasL fusion, the present invention also provides a vector (which may of course be may be a plasmid vector, a viral vector, a baculovirus vector, etc.) for the regulatable expression of FasL or a FasL fusion, comprising a nucleic acid encoding FasL or a FasL fusion operatively linked to a transcription regulatory sequence to be used in the methods of the invention. In one embodiment, the transcription regulatory sequence may be inducible, i.e., expression of FasL or a FasL fusion will not proceed unless the appropriate activator for the particular transcription regulatory sequence is present. In another embodiment, the transcription regulatory sequence may be repressible, i.e., expression of FasL or a FasL fusion will proceed unless the appropriate repressor for the particular transcription regulatory sequence is present.

In yet another embodiment, the vector may additionally comprise a nucleic acid encoding a trans-acting factor which interacts with the transcription regulatory sequence to affect transcription of FasL or a FasL fusion. Where the transcription regulatory sequence is inducible, the trans-acting factor will be an activator. Where the transcription regulatory sequence is repressible, the trans-acting factor will be a repressor.

In a more preferred embodiment, the transcription regulatory sequence is a tet responsive element (TRE), and the trans-acting factor is a tet-responsive transacting expression element (tTA). In the most preferred embodiment, the invention utilizes the vector Ad/FasL-GFP$_{TET}$. This is a replication-deficient adenoviral vector that expresses a fusion of murine FasL and green fluorescent protein (GFP). FasL-GFP retains full activity of wild-type FasL, at the same time allowing for easy visualization and quantification in both living and fixed cells. The fusion protein is under the control of tetracycline-regulated gene expression system. A tight control is achieved by creating this novel "double recombinant" Ad vector, in which the tet-responsive element and the transactivator element are built into the opposite ends of the same vector to avoid

enhancer interference. Expression can be conveniently regulated by tetracycline or any derivative thereof, which includes, but is not limited to, doxycycline, in a dose-dependent manner. The vector efficiently delivers FasL-GFP gene to cells *in vivo and in vitro*, and the expression level of the fusion protein may be modulated by the concentration of doxycycline added to the culture media or administered to the subject. As may be seen in the following examples, Ad/FasL-GFP$_{TET}$, is able to deliver FasL-GFP to transformed and primary cell lines, with the expression of the fusion protein in those cells regulated by varying the level of doxycycline in the media. Amounts of FasL-GFP can be easily detected and quantified through the fluorescence of its GFP component, and correlated with the levels of apoptosis in the target and neighboring cells.

This vector design, which delivers an entire tet-regulated gene expression system, is more efficient and economical than multiple vector strategies, and can be applied to any situation where regulation of protein expression is desired. Accordingly, in another embodiment, the invention relates to a regulatable expression vector comprising a nucleic acid encoding a transactivator protein that binds to a tet-responsive transactivating expression element; and a regulatory element comprising a tet-responsive transactivating expression element; wherein a nucleic acid encoding a protein to be expressed may be inserted downstream of the regulatory element. In a preferred embodiment, the vector is a viral vector. In a more preferred embodiment, the viral vector is an adenovirus vector, and the nucleic acid encoding the transactivator protein and the nucleic acid encoding the regulatory element are oriented at opposite ends of the vector. Of course, the vector may be any other type of viral vector, including but not limited to a vaccinia vector or a retrovirus vector. In another embodiment of this vector, the protein to be expressed is a fused to a reporter, including, but not limited to, green fluorescent protein. For example, a preferred vector for expression of FasL-GFP is synthesized by ligating pLAd-C.tTA and pRAd-TGFsL to a portion of the Ad5 genome (snb 360) to produce the vector Ad/FasL-GFP$_{TET}$ as

described below and as shown in Figures 1A-C. In a most preferred embodiment, the vector is pAd_{TET}, which may be synthesized by removing FasL-GFP from vector pRAd-TGFsL, and ligating the resulting vector to pLAd-C.tTA, in the same way as described for the production of the vector $Ad/FasL\text{-}GFP_{TET}$ in Figure 1A-C. The vector pAd_{TET} can be utilized to express an unlimited variety of heterologous proteins for which tight regulation is desired.

The nucleic acid encoding a FasL or a vector may also contain a selectable marker which can be used to screen for those cells which contain the nucleic acid or vector and which express the selectable marker. In this manner, one can readily separate those cells containing the nucleic acid or the vector and expressing the selectable marker from those cells either containing the nucleic acid or the vector but not expressing the selectable marker, and from those cells not containing the nucleic acid or the vector. The specific selectable marker used can of course be any selectable marker which can be used to select against eukaryotic cells not containing and expressing the selectable marker. The selection can be based on the death of cells not containing and expressing the selectable marker, such as where the selectable marker is a gene encoding a drug resistance protein. An example of such a drug resistance gene for eukaryotic cells is a neomycin resistance gene. Cells expressing a neomycin resistance gene are able to survive in the presence of the antibiotic G418, or Geneticin7, whereas those eukaryotic cells not containing or not expressing a neomycin resistance gene are selected against in the presence of G418. One skilled in the art will appreciate that there are other examples of selectable markers, such as the *hph* gene which can be selected for with the antibiotic Hygromycin B, or the *E. coli Ecogpt* gene which can be selected for with the antibiotic Mycophenolic acid. The specific selectable marker used is therefore variable.

The selectable marker can also be a marker that can be used to isolate those cells containing and expressing the selectable marker gene from those not containing and/or

not expressing the selectable marker gene by a means other than the ability to grow in the presence of an antibiotic. For example, the selectable marker can encode a protein which, when expressed, allows those cells expressing the selectable marker encoding the marker to be identified. For example, the selectable marker can encode a luminescent protein, such as a luciferase protein or a green fluorescent protein, and the cells expressing the selectable marker encoding the luminescent protein can be identified from those cells not containing or not expressing the selectable marker encoding a luminescent protein. Alternatively, the selectable marker can be a sequence encoding a protein such as chloramphenicol acetyl transferase (CAT). By methods well known in the art, those cells producing CAT can readily be identified and distinguished from those cells not producing CAT.

The various vectors and hosts used to express the nucleic acid encoding a Fas ligand may be used to express the nucleic acids *in culture* or *in vitro*. For example, a vector comprising a nucleic acid encoding a Fas ligand may be introduced into a tissue culture cell line, such as COS cells, and expressed whereby the nucleic acid is expressed *in culture*. In this manner, one skilled in the art can select a cell type that may have a limited life in the host organism such that the host can effectively clear the cell expressing the FasL in a period of time such that any possible apoptotic effects on non-target surrounding cells or tissues can be minimized. Alternatively, cells from a subject may be removed from the subject, administered the nucleic acid encoding a Fas ligand, and then replaced into the subject. In this *ex vivo* treatment procedure, the cells can be manipulated to facilitate the uptake of the nucleic acid encoding a Fas ligand without unnecessary adverse effects on the subject.

The various vectors and hosts used to express the nucleic acids of the present invention may be used to express the nucleic acids *in vivo*. For example, a vector comprising a nucleic acid encoding a Fas ligand may be introduced into cells of a eukaryotic host, preferably tumor cells, to treat Fas^+ tumor cells *in situ*. As briefly

discussed above, one skilled in the art will appreciate that specific tissues can be treated by selectively administering the vector to the host. For example, administering an adenovirus vector via an aerosol such as through the use of an inhaler can selectively administer the vector to the lungs. Alternatively, the use of a suppository can be used to selectively administer the vector to cells of the colon. Alternatively, delivering the vector topically such as in a cream can selectively deliver the vector or nucleic acid to skin cells or the cervix. One skilled in the art will recognize the various methods that can routinely be used to selectively deliver the vector, or alternatively, the nucleic acid encoding a Fas ligand, to specific organs or cells. The vectors of the invention, when expressing proteins for treating cancer or other diseases, can be administered in conjunction with (before, during, or after) other therapeutic agents against the cancer or disease to be treated. These agents can be administered at doses either known or determined to be effective and may be administered at reduced doses due to the presence of the vector-expressed protein.

One skilled in the art will also appreciate that delivery can be manually facilitated through such methods as injection of the vector or the nucleic acid to the selected site. For example, direct injection can be used to deliver the vector or nucleic acid to specific brain and/or breast location. In one embodiment of the present invention, direct injection of a nucleic acid or a vector comprising a nucleic acid encoding a Fas ligand is used for delivery into breast tumor masses.

It is contemplated that using the methods and vectors of the present invention, a nucleic acid encoding FasL can be administered to a cell or to a subject, most preferably, humans, to treat disease states, preferably cancer. The present nucleic acid, whether alone, in combination with another compound or composition (e.g., a chemotherapy agent), or as part of a vector-based delivery system, may be administered parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, topically, transdermally, or the like, although topical administration is

typically preferred. The exact amount of such nucleic acids, compositions, vectors, etc., required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the disease or condition that is being treated, the particular compound or composition used, its mode of administration, and the like. Thus, it is not possible or necessary to specify an exact amount. However, an appropriate amount may be determined by one of ordinary skill in the art using methods well known in the art (see, e.g., *Martin et al.*, 1989).

For topical administration, the nucleic acid encoding FasL, compositions thereof, and/or vectors comprising the nucleic acid can be in pharmaceutical compositions in the form of solid, semi-solid or liquid dosage forms, such as, for example powders, liquids, suspension, lotions, creams, gels or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions can typically include an effective amount of the selected nucleic acid, composition, or vector in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected nucleic acid, composition thereof, or vector without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Alternatively or additionally, parenteral administration, if used, is generally characterized by injection e.g., by intravenous injection including regional perfusion through a blood vessel supplying the tissues(s) or organ(s) having the target cell(s). Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Parenteral administration can also employ the use of a slow release or sustained release system, such that a constant level of dosage is maintained (*See*, for

example, U.S. Patent No. 3,710,795). The compound can be injected directly to the site of cells or tissues expressing a Fas^+ phenotype, or they can be injected such that they diffuse or circulate to the site of the Fas^+ phenotypic cells.

Dosages will depend upon the mode of administration, the disease or condition to be treated, and the individual subject's condition. Dosages will also depend upon the material being administered, *e.g.*, a nucleic acid, a vector comprising a nucleic acid, or another type of compound or composition. Such dosages are known in the art. Furthermore, the dosage can be adjusted according to the typical dosage for the specific disease or condition to be treated. Furthermore, culture cells of the target cell type can be used to optimize the dosage for the target cells *in vivo*, and transformation from varying dosages achieved in culture cells of the same type as the target cell type can be monitored. Often a single dose can be sufficient; however, the dose can be repeated if desirable. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication. Examples of effective doses in non-human animals are provided in the Examples. Based on art accepted formulas, effective doses in humans can be routinely calculated from the doses provided and shown to be effective.

For administration to a cell in a subject, the compound or composition, once in the subject, will of course adjust to the subjects body temperature. For *ex vivo* administration, the compound or composition can be administered by any standard methods that would maintain viability of the cells, such as by adding it to culture medium (appropriate for the target cells) and adding this medium directly to the cells. As is known in the art, any medium used in this method can be aqueous and non-toxic so as not to render the cells non-viable. In addition, it can contain standard nutrients for maintaining viability of cells, if desired. For *in vivo* administration, the complex can be

added to, for example, a blood sample or a tissue sample from the patient, or to a pharmaceutically acceptable carrier, e.g., saline and buffered saline, and administered by any of several means known in the art. Other examples of administration include inhalation of an aerosol, subcutaneous or intramuscular injection, direct transfection of a nucleic acid sequence encoding the compound where the compound is a nucleic acid or a protein into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods include oral administration, particularly when the composition is encapsulated, or rectal administration, particularly when the composition is in suppository form. A pharmaceutically acceptable carrier includes any material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Specifically, if a particular cell type *in vivo* is to be targeted, for example, by regional perfusion of an organ or tumor, cells from the target tissue can be biopsied and optimal dosages for import of the complex into that tissue can be determined *in vitro*, as described herein and as known in the art, to optimize the *in vivo* dosage, including concentration and time length. Alternatively, culture cells of the same cell type can also be used to optimize the dosage for the target cells *in vivo*. For example, intratumoral injection amounts and rates can be controlled using a controllable pump, such as a computer controlled pump or a micro-thermal pump, to control the rate and distribution of the nucleic acid or vector in the tumor or tissue. Example 4 demonstrates effective dosages of AdFasL-GFP_{TET} used for in vivo treatment of both breast and brain tumors in mice. One of ordinary skill will readily know how to extrapolate these figures to determine effective human dosages.

For either *ex vivo* or *in vivo* use, the nucleic acid, vector, or composition can be administered at any effective concentration. An effective concentration is that amount that results in killing, reduction, inhibition, or prevention of a transformed phenotype of the cells.

The nucleic acid or vector can be administered in a composition. For example, the composition can comprise other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. Furthermore, the composition can comprise, in addition to the nucleic acid or vector, lipids such as liposomes, such as cationic liposomes (e.g., DOTMA, DOPE, DC-cholesterol) or anionic liposomes. Liposomes can further comprise proteins to facilitate targeting a particular cell, if desired. Administration of a composition comprising a nucleic acid or a vector and a cationic liposome can be administered to the blood afferent to a target organ or inhaled into the respiratory tract to target cells of the respiratory tract. Regarding liposomes, see, e.g., Brigham et al. *Am. J. Resp. Cell. Mol. Biol.* 1:95-100 (1989); Felgner et al. *Proc. Natl. Acad. Sci USA* 84:7413-7417 (1987); U.S. Pat. No.4,897,355. Furthermore, the nucleic acid or a vector can be administered as a component of a microcapsule that can be targeted to specific cell types, such as macrophages, or where the diffusion of the compound or delivery of the compound from the microcapsule is designed for a specific rate or dosage.

Any cell, specifically a tumor cell, which expresses Fas can be treated by the methods of the present invention. Fas is primarily a surface protein and a cell expressing a Fas ligand can be used to treat the Fas-expressing cell by the Fas-Fas ligand induction of apoptosis. Although the cell expressing the Fas ligand can interact with the Fas-expressing cell via interactions of the Fas and the Fas ligands on the surface of the cells, and therefore treat Fas-expressing cells that the Fas ligand - expressing cells can make contact with, the Fas ligand-producing cells can also regulate the Fas-expressing cell by producing soluble Fas ligand which then interacts with Fas

and also induces apoptosis.

The interaction of the Fas and the Fas ligand is typically a ligand-receptor binding, although the interaction does not have to be binding per se, but includes any cellular reaction which results from any interaction of the Fas and the Fas ligand. Therefore any cellular apoptosis via Fas that results from the expression of a Fas ligand by that same cell or a second cell which expresses a Fas ligand is hereby contemplated.

Although any cell expressing Fas can be induced to undergo apoptosis using the methods of the present invention, a preferred embodiment is inducing Fas^+ tumor cells to undergo apoptosis using these methods. In this embodiment, these tumor cells can selectively be induced to undergo apoptosis and then die, thereby treating a tumor. In another preferred embodiment, the tumor is a solid tumor and the tumor is injected with a recombinant virus which can infect the cells of the tumor and thereby cause them to express a Fas ligand, and whereby the interaction of the FasL-expressing cells with the Fas-expressing cells causes the Fas^+ cells to undergo apoptosis. The Fas-expressing cells which are affected by the FasL-expressing cells are typically cells adjacent to the FasL-expressing cells since typically a cell-to-cell contact is necessary for the apoptotic signal be effectuated. The affected Fas cell can be removed from the immediate surroundings of the FasL-expressing cell, however, such as where the FasL-expressing cell has mobilized and/or where the FasL-expressing cell produces soluble FasL. The Fas ligand-expressing cells can also cause their own death if those cells also are Fas^+ cells. In this approach, the methods of the present invention can cause Fas^+ cells to die, but the tumor cells that now express the Fas ligand also will die, thereby eliminating those tumor cells that might otherwise cause regression of the tumor.

The present invention is more particularly described in the following examples which is intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

Example 1

In an example of the methods described above and depicted in Figure 1, a recombinant adenovirus containing a nucleic acid encoding the human Fas ligand was constructed. Additionally, a recombinant adenovirus was constructed containing a nucleic acid encoding the human Fas ligand and also encoding the jellyfish green fluorescent protein (GFP) such that a fusion protein was ultimately translated. This fusion protein was used to monitor the expression and localization of the protein in cultured cells and in animal tissues following transduction with the adenovirus vector.

Three different tumor cell lines were isolated from breast cancer patients, all of which exhibited a high degree of sensitivity to the Fas ligand treatment via the adenovirus vector. This demonstrates the tumor cells could be effectively treated, or killed, using these methods. Parallel experiments also demonstrated several prostate cancer cell lines are extremely sensitive to Fas-mediated apoptosis since complete killing of these cells was obtained using Adenovirus-mediated introduction of a nucleic acid encoding Fas ligand into these cells.

Additionally, six nude mice were implanted with 10^5 breast cancer cells or prostate cancer cells on each side of the animal. When tumor sizes reached approximately 5 mm in diameter, all tumors on one side of the animals were injected with 10^8 pfu of an adenovirus vector containing a nucleic acid encoding a Fas ligand (AdFasL). All tumors on the other side of the animal were injected with 10^8 pfu of a control adenovirus (AdlacZ). At three weeks post-injection, all tumors injected with AdFasL exhibited significant regression of the tumor in comparison with the control-treated tumor. Histological analysis of the residual tumors in some of the mice showed

only infiltrating immune cells and fibroblasts with no apparent cancer cells remaining.

Example 2: Controlled Delivery of a FasL-GFP Fusion Protein with a Complex Adenoviral Vector

Fas ligand (FasL) induces apoptosis in cells that express Fas receptor and plays important roles in immune response, degenerative and lymphoproliferative diseases and tumorigenesis. It is also involved in generation of immune privilege sites and is therefore of interest to the field of gene therapy. We describe the construction and characterization of replication-deficient adenoviral vectors that express a fusion of murine FasL and green fluorescent protein (GFP). FasL-GFP retains full activity of wild-type FasL, at the same time allowing for easy visualization and quantification in both living and fixed cells. The fusion protein is under the control of tetracycline-regulated gene expression system. A tight control is achieved by creating a novel A double recombinant Ad vector, in which the tet-responsive element and the transactivator element are built into the opposite ends of the same vector to avoid enhancer interference. Expression can be conveniently regulated by tetracycline or its derivatives in a dose-dependent manner. The vector was able to efficiently deliver FasL-GFP gene to cells *in vitro*, and the expression level of the fusion protein was modulated by the concentration of doxycycline in culture media. This regulation allows us to produce high titers of the vector by inhibiting FasL expression in a CrmA-expressing cell line. Induction of apoptosis was demonstrated in all cell lines tested. These results indicate that our vector is a potentially valuable tool for FasL-based gene therapy of cancer and for the study of FasL/Fas-mediated apoptosis and immune privilege.

Materials and Methods

Cells: HeLa and 293 cells were obtained from the American Type Culture Collection (ATCC CCL-2.1 and ATCC CRL-1573, respectively) and maintained as monolayers at 37• C under 5% CO_2 in Dulbecco=s modified Eagle=s medium (DMEM;

Gibco BRL) supplemented with 10% bovine calf serum (BCS; HyClone) and 1% penicillin/streptomycin (Cellgro). Cultured rat myoblasts were maintained in H-21 (Cellgro) media supplemented with 20% Fetal Bovine Serum (FBS; HyClone) and 1% each of penicillin/streptomycin and fungizone.

For DNA transfections, 5_10^5 cells per well were seeded on 6-well plates (Greiner) and transfected 24 hours later using LipofectAMINE (Gibco BRL) according to manufacturer=s instructions.

To produce a cytokine response modifier A (CrmA) -expressing 293 cell line, pCrmA-I-Neo was transfected into HEK293 cells. Neo-positive clones were selected by adding G418 to the media at 0.4 g/L for 4 weeks, at the end of which time individual clones were picked up, propagated and assayed for CrmA expression by their resistance to FasL-induced apoptosis.

Construction of plasmids and recombinant adenoviral vectors: Vectors pEGFP-1 and pEGFP1-C1 were obtained from Clontech. They contain a red-shifted variant of wild type green fluorescent protein (wt GFP) gene, with brighter fluorescence and "humanized" codon usage. (Zhang, G., V. Gurtu and S. R. Kain. 1996. "An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells." (Biochem Biophys Res Commun **227**:707-11.) This protein will be referred to as "GFP" in this Example. The mouse FasL cDNA sequence, available in Genbank, was in a Bluescript (Invitrogen) vector. Vectors pUHD10-3 and pUHD15-1 (Gossen, M. and H. Bujard, "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters" Proc Natl Acad Sci U S A **89**:5547-51, 1992) are available from Clontech. GFP-FasL fusion gene was constructed by inserting DNA coding for aa 11 to aa 279 of the murine Fas ligand in-frame downstream of the GFP sequence in pEGFP-C1, to generate pC.GFsl. The fusion gene from pC.GFsl was inserted into pUHD10-3 to produce p10-3.GFsl. Cowpox virus (Chordopoxvirinae) cytokine response modifier

A (crmA; CPV-W2) cDNA in pcDNA3 vector is available from Genentech. The CrmA gene was excised from pcDNA3 and inserted into pIRES-Neo vector (Clontech) to generate pCrmA-I-Neo.

GFP, FasL, FasL-GFP and LacZ genes were cloned into the E1 shuttle vector, pLAd-CMV to generate pLAd-C.Gf, pLAd-C.Fsl, pLAd-C.GFsl and pLAd-C.Lz constructs, respectively (Fig. 1A). The Tet-OFF fusion activator protein expression cassette was extracted from pUHD15-1 and inserted into pLAd-CMVie to generate pLAd-C.rTa. The GFP-FasL fusion gene expression cassette was excised from p10-3.GFsl and inserted into pRAd.mcs, a shuttle vector for transgene insertion between E4 and right ITR of Ad5. The resulting construct was called pRAd-T.GFsl (Fig. 1B).

The assembly of rAd/FasL-GFP$_{TET}$ vector is shown in Figure 1C. Other rAd genomes used in this study were constructed using a similar strategy. All vectors were based on Ad5sub360 (ΔE3) with additional deletion of all E4 ORFs with the exception of ORF6. (Huang, M. M. and P. Hearing. 1989) The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. (Genes Dev 3:1699-710).

Propagation of viral vectors: The 293 cells, which provide Ad5 E1a and E1b functions in trans (Graham, F. L., J. Smiley, W. C. Russell and R. Nairn; "Characteristics of a human cell line transformed by DNA from human adenovirus type 5" (J Gen Virol **36**:59-74,1977), were transfected with the ligation mixture containing the rAd vector DNA using LipofectAMINE method. Transfected cells were maintained until adenovirus-related cytopathic effects (CPE) were observed (typically between seven and 14 days), at which point the cells were collected. Vector propagation and amplification was then achieved by standard techniques. The stocks were titrated on 293 or 293CrmA cells and plaques were scored to determine vector yields as PFU/ml. Vectors were also titrated using GFP fluorescence or X-gal staining,

as appropriate. In both cases, titer estimates were in good agreement with PFU/ml.

Western blot analysis: 10 cm plates (Greiner) were seeded with 10^6 cells of primary rat myoblasts. After 24 hours, plates were infected with rAd/FasL-GFP_{TET} or control vector at multiplicity of infection (MOI) of 2. At 24 hours postinfection, the plates were washed twice with PBS. The cells were collected and lysed in 200 _l of cell lysis buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 2% SDS, 0.1% Bromophenol Blue, 1 mM PMSF (Sigma), 50 µg/ml leupeptin (Sigma), 2 µg/ml aprotinin (Sigma) and 1 ng/ml pepstatin (Sigma). The samples were boiled for 5 minutes and 1/10 of the original amount (10^6 cells) was loaded per lane of an 8% SDS-PAGE minigel (BioRad), which was run at 20 mA for 3 hours. Human recombinant FasL (C-terminal) was obtained from Santa Cruz Laboratories. The proteins were transferred to a nitrocellulose membrane (Pharmacia Biotech) using a semi-dry gel transfer apparatus (BioRad). The membrane was blocked by incubation (2 hours at 37°C) in a solution containing 10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 3% (w/v) BSA, 5% (w/v) powdered milk, 0.2% (v/v) Tween-20 (Amresco, Solon, OH) and 0.02% (w/v) sodium azide (Sigma). The polyclonal rabbit anti-FasL antibody (Santa Cruz) was diluted 1:100 with blocking solution and incubated with the membrane for 2 hours at ambient temperature. The blot was washed with 10 mM Tris-HCl (pH 7.5) and 140 mM NaCl solution twice, then incubated with goat anti-rabbit IgG conjugated with HRPO (Caltag, Burlingame, CA) diluted 1:10000. The blot was developed in ECL reagent (Amersham Life Science) overnight and visualized with Kodak X-ray film.

Detection of apoptosis: Early detection of apoptosis in cultured adherent cells was accomplished by utilizing the In Situ Cell Death Detection Kit, AP (Boehringer Mannheim) according to manufacturers instructions. This kit utilizes the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) process to incorporate fluorescein at free 3'-OH DNA ends and detect it with anti-fluorescein antibody conjugated to alkaline phosphatase. After substrate reaction, stained cells can

be visualized using light microscopy.

Results:

Functional analysis of FasL and FasL-GFP proteins: In order to demonstrate that the Fas ligand-GFP (FasL-GFP) fusion protein retains full FasL activity, we have analyzed and compared the function of the FasL and FasL-GFP proteins by using transient DNA transfections into cells susceptible to Fas-mediated apoptosis. Triplicates of wells of HeLa cells were transfected with vectors expressing FasL, GFP-FasL or • -galactosidase as a control. At 24 hours post-transfection, cells were fixed and analyzed for apoptosis by using the TUNEL kit. Typically, transfection efficiencies between 10 and 25% were achieved as determined by X-Gal staining of cells transfected with pcDNA3-LacZ. Large numbers of HeLa cells transfected with vectors expressing either FasL or FasL-GFP showed typical apoptotic morphology (such as membrane blebbing and loss of adherence) and stained positive in the TUNEL assay. Very few cells transfected with a control plasmid underwent apoptosis. The numbers of apoptotic cells in wells transfected with FasL-GFP vector were reproducibly similar to those transfected with FasL vector, suggesting that the wild-type and fusion proteins have comparable activity.

Construction and characterization of adenoviral vectors: Our goal was to produce large amounts of adenoviral vectors in which the FasL expression could be regulated. This regulation allows control of the levels of FasL expression in target cells and thus facilitates the study of its biological effects. In addition, amplification of rAd vectors constitutively expressing FasL or FasL-GFP in 293 cells would be expected to produce low titers Muruve, D. A., A. G. Nicolson, R. C. Manfro, T. B. Strom, V. P. Sukhatme and T. A. Libermann. 1997. Adenovirus-mediated expression of Fas ligand induces hepatic apoptosis after Systemic administration and apoptosis of ex vivo-infected pancreatic islet allografts and isografts. Hum Gene Ther **8**:955-63. because

FasL expression causes apoptosis of the virus-producing cells. To achieve the controlled FasL-GFP expression, we designed the rAd/FasL-GFP$_{TET}$ vector in which the FasL-GFP is expressed from a TRE promoter Gossen, M. and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. Proc Natl Acad Sci U S A **89**:5547-51. We inserted CMVie promoter-driven tTA gene (the "tet-off" element) into the Ad5 E1 region and the TRE-controlled FasL-GFP fusion gene near the right ITR. This strategy was based on the following considerations. First, this strategy delivers the entire tet-regulated expression system using a single vector, rather than using two Ad vectors as have been described previously Harding, T. C., B. J. Geddes, D. Murphy, D. Knight and J. B. Uney. 1998. Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system [see comments]. Nat Biotechnol **16**:553-5. Use of a single vector allows a more efficient delivery to target cells as well as a more uniform regulation of protein expression. This strategy also achieves maximum possible separation between the enhancer elements of the CMVie promoter and the TRE promoter, in order to minimize background (unregulated) expression of FasL-GFP protein (Fig. 1B and 1C). By placing the TRE promoter at the right end of the Ad5 genome, a similar result was obtained with respect to the E1A enhancer elements, which are located within the Ad5 packaging signals Hearing, P. and T. Shenk. 1983. The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. Cell **33**:695-703.. These elements have been reported to interact with some promoters cloned into the E1 region Shi, Q., Y. Wang and R. Worton. 1997. Modulation of the specificity and activity of a cellular promoter in an adenoviral vector. Hum Gene Ther **8**:403-10.

The genomes of recombinant adenoviral vectors used in this study were assembled *in vitro* in large-scale ligation reactions as schematically diagrammed in Figure 1C. These genomes were then gel-purified and transfected into 293 cells and the resulting cultures were propagated until virus-induced CPE was observed. In the case of vectors expressing β-galactosidase or GFP, CPE occurred at significantly earlier time

points than for vectors expressing FasL or FasL-GFP, indicating that adenoviral vector replication was likely deleteriously affected by FasL activity. Primary vector stocks were amplified according to established techniques, and rAd DNA was extracted and examined for structural integrity by restriction enzyme digests.

The titers of rAd/FasL and rAd/FasL-GFP_{TET} in 293 cells were typically 30 to 100-fold lower then titers of rAd/LacZ or rAd/GFP. Comparison of titers of rAd vectors with FasL activity demonstrated a substantial improvement (between 8- and 12-fold) in the yield of these vectors when they were produced in 293CrmA cells (Fig. 2). Amplification of the control vector rAd/LacZ in either 293 or 293CrmA cells resulted in essentially the same yield (Fig. 2). Subsequently, generation and amplification of all vectors with FasL activity was carried out in 293CrmA cells.

Induction of apoptosis by adenovirus-mediated FasL expression: To functionally demonstrate that adenovirus-mediated FasL expression, we transduced HeLa cells with rAd/FasL-GFP_{TET} at different MOI. At 24 hours post-transduction, cells were analyzed for apoptosis. Cells infected with rAd/FasL-GFP_{TET} demonstrated typical apoptotic morphology. The numbers of apoptotic cells increased with the increasing vector titers. In contrast, plates transduced with the control vector rAd/LacZ at the same MOI did not generate apoptotic cells in excess of untransduced controls. The overall efficiency of transduction was determined by X-gal staining and shows increasing numbers of _-galactosidase-positive cells with increasing MOI. We have observed that the numbers of apoptotic cells are noticeably higher than those of the cells with detectable GFP fluorescence, or of the X-gal stained cells transduced at the same. Thus, apoptosis of cells not infected with the vector, but adjacent to the cells that are, is caused by the interactions of FasL on the surface of infected cells with Fas receptors on their neighbors.

Detection and cellular localization of FasL-GFP fusion protein: Wild-type

FasL is a type II membrane protein. To demonstrate that the FasL-GFP fusion protein is also targeted to cellular membrane, we took advantage of the fluorescence of its GFP component, which can be detected in living cells using a fluorescent microscope with a FITC filter set. We have used this technique to observe the expression and cellular localization of our FasL-GFP fusion protein when expressed from rAd vector. In HeLa cells, expression of FasL-GFP causes apoptosis at protein levels close to the detection threshold of GFP. Therefore, the expression of FasL-GFP was analyzed in primary rat myoblasts, which we found to be relatively resistant to FasL-induced apoptosis. High levels of FasL-GFP expression can be detected in myoblasts at 24 hours post-infection with rAd/FasL-GFP$_{TET}$ at MOI of 10. Membrane-associated expression of FasL-GFP is evident in the majority of the transduced cells. In contrast, the fluorescence pattern of GFP itself is evenly distributed in the cytoplasm of the cells, while often being excluded from the nucleus. These localization differences are also apparent in transduced 293CrmA cells at higher magnification. These results indicate that the FasL-GFP fusion protein is directed to the cell surface, where it can interact with the Fas receptor in a manner analogous to that of wt FasL.

Regulation of FasL-GFP expression from rAd vector: To show that the present vector has the ability to regulate the amount of FasL activity produced by our rAd vector in target cells, we have performed experiments to establish the levels of FasL expression under induced or uninduced conditions at both the levels of protein synthesis and function. In rAd/FasL-GFP$_{TET}$ vector, expression of FasL-GFP fusion protein is designed to be activated by the binding of the tetR-VP16 fusion protein (constituatively expressed from the same vector; see Fig. 1C) to the heptamer of tet-operators upstream of a minimal CMVie promoter Gossen, M. and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. Proc Natl Acad Sci U S A 89:5547-51.. Presence of doxycycline in the cell should inhibit this binding - and therefore the expression of FasL-GFP - in a concentration-dependent manner.

First, we determined the amounts of FasL-GFP produced in transduced cells by using Western blot analysis. We infected primary rat myoblasts with rAd/FasL-GFP$_{TET}$ at an MOI of 2 and cultured these cells in the absence or presence of doxycycline, a tetracycline derivative. Low MOI was chosen to maximize number of cells transduced with a single copy of the vector. After 48 hours, cells were lysed and the lysates analyzed by Western blotting using a polyclonal antibody against the extracellular domain of FasL. A single specific band larger than the predicted size of wt FasL was detected. The intensity of the band decreased with the increasing concentration of doxycycline, and no band could be detected in the cell lysates that have been cultured in the presence of 0.5 mg/L or higher concentration of doxycycline. No FasL-specific band was observed in cells transduced with a control vector. No bands of lesser size, corresponding to the breakdown or cleavage products, were detected either in the cell lysates or in the media supernatant. These results indicate that the amount of GFP-FasL protein produced in the cell from the rAd/FasL-GFP$_{TET}$ vector can be regulated by the concentration of doxycycline in culture medium, and that this protein is stable and does not undergo appreciable cleavage once on the cell surface.

We have also analyzed the regulation of FasL activity, i.e. the induction of apoptosis in Fas-positive target cells. Wells of HeLa cells were transduced with rAd/FasL-GFP$_{TET}$ at an MOI of 2 and cultured in the presence of various concentrations of doxycycline. At 24 hours post-transduction, cells were analyzed for apoptotic phenotype. The results confirm that the induction of apoptosis in cells transduced with rAd/FasL-GFP$_{TET}$ can be regulated by doxycycline.

In the regulated protein expression system that we chose, presence of doxycycline inhibits the binding of tTA to TRE and turns off FasL-GFP transcription in a dose-dependent manner. We elected to insert the constitutively expressed activator into the E1 region and the FasL-GFP expression cassette into a novel cloning site between the E4 promoter and the right ITR of Ad5, reasoning that this arrangement

would minimize the effect of the E1A enhancer present within the packaging region of adenovirus and the CMVie enhancer within the tTA promoter on the TRE, and thus reduce background expression of the fusion protein in the presence of inhibitor. This system performed successfully in the context of adenoviral vector, such that the expression of FasL-GFP could be efficiently regulated by varying the doxycycline concentrations in cell culture medium.

In the course of our experiments, we have observed that 293 cells are susceptible to FasL-induced apoptosis. This effect acts to significantly limit the titers of rAd vectors expressing FasL. This is true even if regulated or tissue-specific promoters are used to express FasL protein, since high levels of protein expression are unavoidable in the course of vector replication in 293 cells. In order to overcome this problem, we have generated a 293 cell line which constitutively expresses CrmA. This protein acts specifically to inhibit the activity of regulatory caspases, which are integral to the Fas apoptosis pathway. By producing our FasL-containing vectors in these cells, we have obtained significant improvements in the vector titers.

In summary, we have developed and tested a rAd vector that expresses a novel FasL-GFP fusion protein under the control of tetracycline-regulated gene expression system. This vector combines high titers and efficient transgene delivery to multiple types of dividing and non-dividing cells with convenient regulation of protein expression and easy detection of the fusion protein in both living and fixed cells. This vector is a valuable tool for treating disease through immunology, transplantation and cancer therapy.

Example 3: Bystander Gene Therapy Using Adenoviral Delivery of a Fas Ligand Fusion Gene

This example describes a type of bystander gene therapy utilizing a Fas Ligand-fusion gene approach that induces prostatic adenocarcinoma to undergo apoptosis

(programmed cell death) through a paracrine/autocrine mechanism. This work provides a novel and potent therapy for treatment of prostate cancer (PCa). Furthermore, specificity for the prostate or any other tissue may be achieved using tissue-specific promoters to allow parenteral delivery of virus for treatment of metastatic disease.

Our therapeutic approach is to deliver and express a Fas Ligand (CD95L-fusion gene) with a second generation adenovirus deleted for E1A, E3 and E4. CD95L expression is controlled by a Tet operator allowing for doxycycline regulation *in vitro* and *in vivo*. The CD95L used in this proposal is the mouse CD95L cDNA truncated by 10 amino acids at its N terminus and fused in frame with a four amino acid linker to the C terminus of an enhanced GFP.

Table 1 presents our data using five PCa cell lines and generally confirms literature reports (Hedlund et al. The Prostate 36:92-101, 1998 and Rokhlin et al. Can. Res. 57:1758-1768, 1997) that demonstrate PCa cell lines are resistant to CH-11 agonist activity. In contrast, we now demonstrate sensitivity to AdGFP-FasL and C2-ceramide in all five PCa cell lines tested to date.

Percent cytotoxicity was determined using the MTS assay. In brief, cells were seeded in a 12-well plate with 1ml of media. Prior to treatments, cells were grown to 75% confluency and treated with either 500ng/ml CH-11 anti-Fas antibody, 500ng/ml Normal Mouse Serum or 30• M C2-ceramide. For adenoviral transduction, approximately $1x10^5$ cells/well were treated with either AdCMVGFP or $AdGFPFasL_{TET}$ at an MOI between 10-1000. For each cell line, positive controls were left untreated, and 1 ml of media was used as a negative control. The cells were incubated for 48 hours at 37_C for maximal cell killing. Media was aspirated and replaced with 0.5ml fresh media + 100_l of CellTiter 96^7 AQueous One Solution Reagent per well. Cells were incubated for an additional 1-3 hours at 37_C. After incubation, 120_l of media was placed into a 96 well plate and absorbance readings were taken using a Vmax

kinetic microplate reader at 490nm. Percent cytotoxicity was calculated as follows: % cytotoxicity = [1-(OD of experimental well/ OD of positive control well)] x 100. For ceramide assays, $1x10^4$ cells/well were seeded in a 96-well plate. The following morning cells were washed and incubated with 100• l of 30• M Dihydro- or C2-ceramide (diluted from a 10mM stock in ethanol) in serum-free RPMI 1640. After 24 hours, 20• l Celltiter 967 Aqueous One Solution Reagent was added to each well and plates were incubated an additional 1-4 hours. Absorbance and % cytotoxicity were determined as above. In each experiment , data points were run in triplicate.

Results:

Clearly, the five PCa cell lines analyzed in Table 1 are largely insensitive to CH-11. Sensitivity to C2-ceramide is relatively uniform at the 30 μM dose suggesting that the apoptosis pathway is intact. Most importantly, all the cell lines are responsive to AdGFP-FasL administration with DU145 being the least sensitive.

Several important points are made by these experiments. First, we show using FACS analysis that CD95 (Fas receptor) was expressed on all candidate PCa cell lines, for all lines we used. Second, we show that the fas receptor blocking antibody (ZB4) does not prevent induction of apoptosis by AdGFP-FasL. We have performed this experiment several times with different doses of ZB4, always with the result that the virus induced the same extent of apoptosis in the presence or absence of the antibody. This suggests that newly synthesized CD95-CD95L may interact perhaps in the golgi (Bennett et al. Science 282:290-293, 1998), on the way to the plasma membrane, or on arrival at the cell surface as a preformed and functional apoptotic signaling complex. Third, our results show that there is no intrinsic property of the adenovirus that facilitated induction of apoptosis in PCa. This was demonstrated by infecting PCa with control virus (AdCMVGFP) plus CH-11 at 500ng/ml. The result was that CH-11 still failed to induce apoptosis. These results show that apoptosis only occurs in $CD95^+$-CH-11 resistant PCa cell lines when viral

directed intracellular expression of CD95L occurs and this was not virus-dependent.

The final and most relevant piece of information pertains to whether we can administer AdGFP-FasL$_{TET}$ without lethality to the subject. This is critically important because a dose as low as $2x10^8$ pfu of virus kills the mouse when administered parenterally. To address this issue, xenografts of PPC1 were developed in Balbc nu/nu mice and treated with various doses of AdCMVGFP control or AdGFP-FasL virus. From these single dose studies, we have evidence that tumor cell growth is retarded or stopped. Further, out of 14 animals treated with virus, none have died from the virus. In summary, we conclude that the GFP-FasL fusion protein in our Ad5 delivery system has strong therapeutic potential for treating PCa.

Development of a version of AdGFP-FasL that is up-regulated by doxycycline.

Our present virus is designed to be administered orthotopically to PCa. If the virus escapes the tumor and enters the body it could be lethal if sufficient virus reaches the reticuloendothelial system (mostly the liver). By administration of doxycycline (dox), expression of CD95L from AdGFP-FasL can be down-regulated, and this danger avoided. A viral vector induced by doxycycline that exhibits "very low" basal activity is constructed by using the Tet regulatable elements set forth in Example 1. This vector is completely repressed relative to GFP-FasL expression in the absence of dox and induced starting at 10ng/ml with maximal induction between 100-500ng/ml. These are easily achievable doses in humans (1-3• g/ml at typical dosage levels). Should adverse effects be observed, dox administration is terminated. However, doxycycline has a serum half-life of 16 hours which we believe argues that the addition of dox to down-regulate expression of Fas Ligand may be better for treating adverse effects in patients since we can rapidly achieve effective doxycycline doses within minutes by parenteral administration. However, the half-life of GFP-FasL is also a factor in this and will be determined experimentally in

Experimental Group B and C. If necessary, addition of a PEST signal can speed degradation (see Clontech catalogue).

Methods:

From a technical standpoint this is straightforward molecular biology. We replace our current Tet repressor and operator system with the rTS^{kid} B/C and rtTA system (Freundlieb et al. J. Gene Med. 1:4-12, 1999). It has already been pointed out that we can place our prostate specific promoters (PSA, PSADBam, PB and ARRPB2, Appendix) into the virus (replacing CMVie) to achieve tissue specificity where only prostate epithelial cells will be able to regulate rtTA. All viruses are grown by standard techniques from 3X plaque-purified samples assessed to be negative for wild-type adenovirus by PCR. All viruses are grown in the presence of 1μg/ml doxycycline in the HEK 293 packaging cell line that constitutively expresses the cowpox virus cytokine response modifier, crmA Rubinchik et al. This is necessary to prevent GFP-FasL induced apoptosis in the packaging cell line. Virus is always purified by isopycnic centrifugation on CsCl, desalted by chromatography, concentrated by filtration and stored frozen in PBS 10% glycerol in small aliquots at –80°C. Virus is thawed only once and administered to the animals under anesthesia, by infusion as described above, at 15ul/min or via the tail vein with a tuberculin syringe. Tumor and animal tissues are collected for frozen sections or, fixed and embedded, where appropriate, and analyzed by H & E, by tunel assays for apoptosis, and by immunostaining to determine neutrophil infiltration and GFP expression where relevant.

Testing the original AdGFP-FasL$_{Tetd}$ (dox down-regulated) on prostate cancer xenografts in Balbc nu/nu mice. These experiments are carried out to establish both toxicological and efficacy parameters. Specifically, we infuse increasing doses $1x10^9$ - $5x10^{10}$ pfu AdGFP-FasL$_{Tetd}$ into 75 to 100 mm^3 tumors to determine: A) lowest successful

dose required to decrease tumor volume by 75% or more following orthotopic administration of virus with one dose and with three doses administered every four days. Tumors are developed from CD95L sensitive PPC1, intermediately sensitive LnCAP C2-4, and more resistant Du145 cell lines. Other parameters of administration are developed based on results with the endpoint always being tumor remission. B) Highest tolerated viral dose following orthotopic administration (up to 5 x 10^{10} pfu). C) Determine if tumor will reoccur at a later time (6-12 months) in the same or distant site (C4-2). D) Highest dose administered i.v. (tail vein) that 50% of mice survive. E) Using data from D, test the effect of doxycycline administration on the animal survival curve and duration of doxycycline protection (Balbc nu/nu mice have no CTL response so adenovirus may survive for a long time). Statistical analysis using a one sided t-test is employed. F) Determine the half-life of GFP-FasL in K562 cells (CD95L resistant, see Table 1) by monitoring GFP (as the GFP-FasL fusion) over time in the presence of 1μg/ml dox using FACS analysis.

The same set of experiments as in B1 above is carried out with the Tet inducible virus constructed as described above.

Toxicology testing of AdGFP-FasL$_{Tetu}$ (upregulated) and AdGFP-FasL$_{Tetd}$ (down-regulated) administered to normal laboratory beagles. Although there are a number of animal models for PCa, none but the dog model well-represent human disease in pathology and anatomy. It has recently been shown that human AdRSVbgal (serotype 5) adenovirus will infect dog epithelial cells, including prostate tumor cells, both *in vitro* and *in vivo* Andrawiss et al. Prostatic Can. Prostatic Dis. 2:25-35, 1999. Comparison of the present AdGFP-Fas$_{Tet}$ in dogs (immunocompetent) verses immunocompromised mice (Balbc nu/nu) provides additional support for a human phase I trial of this gene therapy approach.

In the following section, experiments are carried out on sexually mature normal dogs to see if orthotopic delivery AdGFP-FasL to hormonal prostate is safe with minimal or no collateral damage.

Purified concentrated adenovirus (AdGFP-FasLboth up- and down-regulated and a reporter virus AdCMVLacZ all serotype 5) is injected via an abdominal surgical approach into one lobe of the dog prostate. This approach is preferable to transrectal introduction because it is believed that direct visualization of the prostate provide for a more accurate introduction of virus in these first series of experiments. Second, because of the highly vascular nature of the dog prostate direct visualization allows us to seal the needle track with topical tissue glue and digital pressure to prevent viral leakage from the injection site. Based on these results , a 3D ultrasound guided transrectal introduction is used to mimic one of the proposed human approaches.

Virus dosages of $5x10^9$, $1x10^{10}$, and $5x10^{10}$ in a constant 400ul volume are used: one set of 2 dogs receives AdCMV-LacZ at $5x10^{10}$ pfu to allow histochemical monitoring of viral spread. Dogs are monitored closely the first 72 hours for any signs of distress. Feces is collected and analyzed for viral shedding by PCR. Urine is also collected by foley catheter and assayed on 293 cells for shed virus and by PCR. At day 7 (2 dogs per viral dose) are euthanized with sodium pentobarbital and processed as described. (Andrawiss et al. Prostatic Can. Prostatic Dis. 2:25-35, 1999). Samples of all tissues are frozen in OCT while the remainder are either fixed and processed for histology (tunel, immunohistochemistry), or stored frozen at -80• C for DNA extraction and PCR using viral-specific primers. Expression of LacZ is examined in the AdCMV-LacZ group to monitor systemic viral spread.

Example 4: Intratumoral Introduction of AdGFP-FasL_{TET} Suppresses Breast Tumor and Brain Tumor Growth in Mice

In this experiment, we implanted 10^6 MCF-7 cells bilaterally into Balbc nu/nu mice. When tumor sizes reached 5 mm in diameter, we infused at 15 µl per minute, 2 X 10^9 pfu AdGFP-FasL_{TET} into the tumors on the right side of the mouse or 2 X 10^9 pfu AdLacZ into the left side, over a period of 10 minutes using a Harvard infusion pump. Tumor

suppression was 80-100% in treated tumors as compared to untreated tumors. In contrast, all tumors injected with AdLacZ grew to about 2 cm in diameter at three weeks after implantation. This demonstrates that FasL-induced apoptosis may be used as a novel treatment for breast cancer.

Similarly, we implanted 10^6 SF767 cells bilaterally into Balbc nu/nu mice. When tumor sizes reached 5 mm in diameter, we infused at 15 µl per minute, 2 X 10^9 pfu AdGFP-FasL_{TET} into the tumors on the right side of the mouse or 2 X 10^9 pfu AdLacZ into the left side, over a period of 10 minutes using a Harvard infusion pump. Tumor suppression was 80-100% in treated tumors as compared to untreated tumors. In contrast, all tumors injected with AdLacZ grew to about 2 cm in diameter at three weeks after implantation. This demonstrates that FasL-induced apoptosis may be used as a novel treatment for brain cancer.

Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

TABLE 1: Fas-Mediated Cytotoxicity in Prostate Cancer Cell Lines Treated for 48 hours with either Anti-Fas Antibody, C2-Ceramide (22 hours) or AdGFP-FasL_{TET} - (expressed as % cytotoxicity _ SD)

Cell Line	Normal Mouse Serum (500 ng/ml)	C2-Ceramide (30 ±M for 22 hrs)	Anti-Fas IgM (CH-11)(500 ng/ml)	AdCMVGFP (MOI 100)	AdGFP-FasL_{TET} (MOI 100)
DU145	0.8 ± 7.1	61 ± 5	6.0 ± 10.4	1.9 ± 2.869.6± 4.5	
PC-3	1.3 ± 5.8	76 ± 9	1.3 ± 2.2	0.9 ± 5.084.8± 1.1	
PPC-1	2.3 ± 0.3	58 ± 7	29.2 ± 2.3	2.3 ± 6.1*	98.0 ± 7.1*
LNCaP	7.5 ± 14.2	ND	11.6 ± 13.7	1.6 ± 3.296.4 ± 4.3	
TSU-Pr1	-3.5 ± 2.3	72 ± 9	-1.9 ± 2.8	11.6 ± 7.0	81.3 ± 5.0
Jurkat(+ctrl)	2.1 ± 5.3	98 ± 2	72.3 ± 0.9	-19.5 ± 22.5^{H}	93.0 ± 3.4^{H}
K-562(-ctrl)	-		-	-1.3± 5.5^{H}	-11.4 ± 8.1^{H}

*MOI 10, ^{H} MOI 1000. In all experiments N=3 (except N=2 for Ceramide experiments using TSU and PC-3). Percent cytotoxicity was determined using the MTS assay. In brief, cells were seeded in a 12-well plate with one ml of media. Prior to treatments, cells were grown to 75% confluency and treated with either 500 mg/ml CH-11 anti-Fas antibody, 500 ng/ml Normal Mouse Serum or 30 μM C2-ceramide. For adenoviral transduction, approximately 1x 10^5 cells/well were treated with either AdCMVGFP or AdGFPFasL_{TET} at an MOI between 10-1000. For each cell line, positive controls were left untreated, and 1 ml of media was used as a negative control. The cells incubated for 48 hours at 37° C for maximal cell killing. Media was aspirated and replaced with 0.5ml fresh media + 100μl of CellTiter 96 AQueous One Solution Reagent per well. Cells were incubated for an additional 1-3 hours at 37° C. After incubation 120μl of media was placed into a 96 well plate and absorbance readings were taken using a Vmax kinetic microplate reader at 490nm. Percent cytotoxicity was calculated as follows: % cytotoxicity = [1-(OD of experimental well/ OD of positive control well)] x 100. For ceramide assays, 1x 10^4

cells/well were seeded in a 96-well plate. The following morning cells were washed and incubated with 100µl of 30µM Dihydro- or C2-ceramide (diluted from a 10mM stock in ethanol) in serum-free RPMI 1640. After 24 hours 20µl Celltiter 96 AQueous One Solution Reagent was added each well and plates were incubated for an additional 1-4 hours. Absorbance and % cytotoxicity were determined as above. In each experiment, data points were run in triplicate.

What is claimed is:

1. A method of killing a Fas^+ tumor cell comprising introducing into a second tumor cell a nucleic acid encoding a Fas ligand (FasL), whereby the second tumor cell expresses the nucleic acid thereby producing FasL, and whereby interaction of the Fas^+ tumor cell with the second tumor cell expressing FasL causes the Fas^+ tumor cell to undergo apoptosis, thereby killing the Fas^+ tumor cell.

2. The method of claim 1, wherein the FasL is membrane-associated.

3. The method of claim 2, wherein the interaction of the Fas^+ tumor cell with the second tumor cell expressing FasL comprises cell-to-cell interaction.

4. The method of claim 1, wherein the tumor is a solid tumor.

5. The method of claim 1, wherein the FasL is a fusion protein.

6. The method of claim 5, wherein the fusion protein comprises FasL and green fluorescent protein.

7. The method of claim 5, wherein the fusion protein comprises FasL and a regulatory protein.

8. The method of claim 1, wherein the nucleic acid encoding FasL also contains a regulatory region which is capable of controlling the expression of the FasL-encoding sequence.

9. The method of claim 8, wherein the regulatory region comprises the Tet-operon.

10. The method of claim 1, wherein the nucleic acid encoding a Fas ligand (FasL) is introduced into the second tumor cell via a vector.

11. The method of claim 10, wherein the vector is a viral vector.

12. The method of claim 11, wherein the viral vector is an adenovirus vector.

13. The method of claim 11, wherein the viral vector is a vaccinia vector.

14. The method of claim 11, wherein the viral vector is a retrovirus vector.

15. The method of claim 1, wherein the FasL is expressed using a tissue-specific promoter.

16. The method of claim 15, wherein the tumor cell is a prostate tumor cell and the tissue-specific promoter comprises a prostate-specific promoter.

17. The method of claim 15, wherein the tumor cell is a breast tumor cell and the tissue-specific promoter comprises a breast-specific promoter.

18. The method of claim 15, wherein the tumor cell is a colon tumor cell and the tissue-specific promoter comprises a colon-specific promoter.

19. The method of claim 15, wherein the tumor cell is a brain tumor cell and the tissue-specific promoter comprises a brain-specific promoter.

20. The method of claim 15, wherein the tumor cell is a kidney tumor cell and the tissue-specific promoter comprises a kidney-specific promoter.

21. The method of claim 15, wherein the tumor cell is a bladder tumor cell and the tissue-specific promoter comprises a bladder-specific promoter.

22. The method of claim 15, wherein the tumor cell is a lung tumor cell and the tissue-specific promoter comprises a lung-specific promoter.

23. The method of claim 15, wherein the tumor cell is a liver tumor cell and the tissue-specific promoter comprises a liver-specific promoter.

24. The method of claim 15, wherein the tumor cell is a thyroid tumor cell and the tissue-specific promoter comprises a thyroid-specific promoter.

25. The method of claim 15, wherein the tumor cell is a stomach tumor cell and the tissue-specific promoter comprises a stomach-specific promoter.

26. The method of claim 15, wherein the tumor cell is a ovarian tumor cell and the tissue-specific promoter comprises a ovary-specific promoter.

27. The method of claim 15, wherein the tumor cell is a cervical tumor cell and the tissue-specific promoter comprises a cervix-specific promoter.

28. The method of claim 15, wherein the prostate-specific promoter is selected from the group consisting of PSA, ΔPSA, ARR2PB, and PB.

29. The method of claim 1, wherein the method is performed *ex vivo*.

30. The method of claim 1, wherein the method is performed *in vivo*.

31. The method of claim 1, wherein the method is performed *in vitro*.

32. A regulatable expression vector comprising a nucleic acid encoding

(A) a transactivator protein that binds to a tet-responsive transactivating expression element; and

(B) a regulatory element comprising a tet-responsive transactivating expression element;

wherein a nucleic acid encoding a protein to be expressed may be inserted downstream of the regulatory element.

33. The vector of claim 32, wherein the vector is a viral vector.

34. The viral vector of claim 33, wherein the viral vector is an adenovirus vector, and wherein the nucleic acid the transactivator protein and the nucleic acid encoding the regulatory element are oriented at opposite ends of the vector.

33. The method of claim 33, wherein the viral vector is a vaccinia vector.

34. The method of claim 33, wherein the viral vector is a retrovirus vector.

33. The vector of claim 32, wherein the protein to be expressed is a fused to a reporter.

34. The vector of claim 33, wherein the reporter is green fluorescent protein.

35. The vector of claim 34, which is pAd_{TET}.

36. A method of killing a Fas^+ tumor cell comprising introducing into a second tumor cell the vector $Ad/FasL\text{-}GFP_{TET}$, whereby the second tumor cell expresses FasL, and whereby interaction of the Fas^+ tumor cell with the second tumor cell

expressing FasL causes the Fas^+ tumor cell to undergo apoptosis, thereby killing the F^+ tumor cell.

37. A vector for the regulated expression of FasL, comprising a nucleic acid encoding FasL operatively linked to a transcription regulatory sequence.

38. The vector of claim 37, wherein the transcription regulatory sequence is inducible.

39. The vector of claim 37, wherein the transcription regulatory sequence is repressible.

40. The vector of claim 37 which is a viral vector.

41. The vector of claim 40 which is an adenovirus vector.

42. The vector of claim 40 which is a vaccinia vector.

43. The vector of claim 40 which is a retrovirus vector.

44. The vector of claim 37 wherein the transcription regulatory sequence is a tet responsive transactivator expression element.

45. The vector of claim 44, wherein the vector additionally comprises a nucleic acid encoding a transactivator protein that interacts with a tet-responsive transactivator expression element

46. The vector of claim 45, which is Ad/FasL-GFP_{TET}.

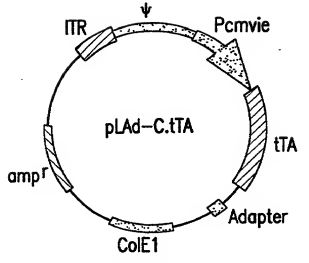
ITR
Ψ
Pcmvie
pLAd–C.tTA
tTA
ampr
Adapter
ColE1

FIG.1A

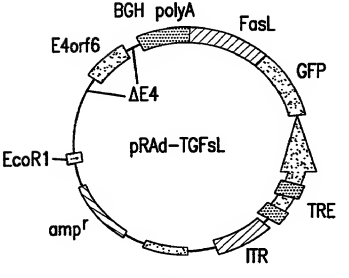
BGH polyA
FasL
E4orf6
GFP
ΔE4
pRAd–TGFsL
EcoR1
TRE
ampr
ITR

FIG.1B

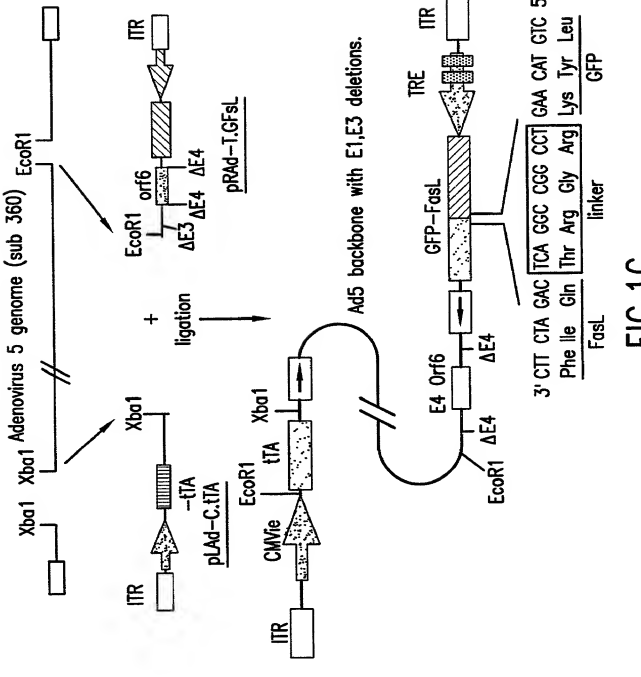
Xba1
Xba1
Adenovirus 5 genome (sub 360)
EcoR1
ITR
Xba1
-tTA
pLAd-C.tTA
EcoR1
tTA
Xba1
CMVie
ITR
+
ligation
EcoR1
orf6
ITR
ΔE3
ΔE4
ΔE4
pRAd-T.GFsL
Ad5 backbone with E1,E3 deletions.
E4 Orf6
ΔE4
EcoR1
GFP-FasL
TRE
ITR
3' CTT CTA GAC TCA GGC CGG CCT GAA CAT GTC 5'
Phe Ile Gln Thr Arg Gly Arg Lys Tyr Leu
FasL
linker
GFP

FIG.1C

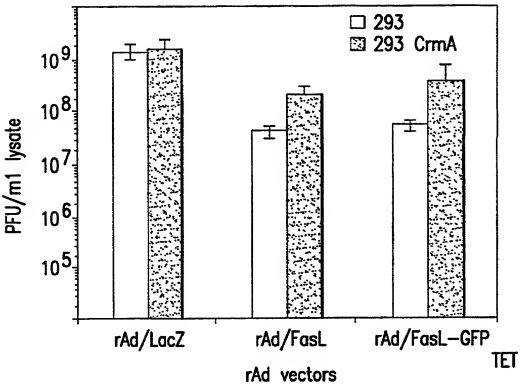
293
293 CrmA
PFU/ml lysate
10^9
10^8
10^7
10^6
10^5
rAd/LacZ
rAd/FasL
rAd/FasL–GFP
TET
rAd vectors

FIG.2

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (12-97)
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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

Attorney Docket Number	22488-710
First Named Inventor	Jian-yun Dong et. al.
COMPLETE IF KNOWN	
Application Number	09/600,521
Filing Date	November 5, 1999
Group Art Unit	Unknown
Examiner Name	Unknown

As a below named Inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD AND COMPOSITION FOR TREATING TUMORS THROUGH FAS LIGAND-INDUCED APOPTOSIS

(Title of the Invention)

the specification of which
☐ is attached hereto
OR
☒ was filed on (MM/DD/YYYY) 11/05/99 as United States Application Number or PCT International Application Number 09/600,521 and was amended on (MM/DD/YYYY) ☐ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/028 attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(h) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
60/107,363	11/06/98	☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/028 attached hereto.

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Please Type a plus sign (+) inside this box → +

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DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of the application is not designated in the prior United States or PCT international application in the manner provided by the first paragraph of 31 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/US99/26221	11/05/1999	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/028 attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☒ Customer Number 021971 → *Place Customer Number Bar Code Label here*

OR

☒ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
Shirley Chen	44,608		

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number or Bar Code Label 021971 *OR* ☐ Correspondence address below

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Country	U.S.	Telephone	650-493-9300			Fax	650-493-6811

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))	Family Name or Surname
JIAN-YUN	DONG

Inventor's Signature						Date	7/20/01
Residence: City	Mount Pleasant	State	SC	Country	US	Citizenship	CN
Post Office Address	1326 Chrismill Lane, Mount Pleasant, SC 29466						
Post Office Address							
City	Mount Pleasant	State	SC	ZIP	29466	Country	US

☒ Additional inventors are being named on the ___ supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto:

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 2 of 3

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle (if any)			**Family Name or Surname**				
JAMES S.			NORRIS				
Inventor's Signature						**Date**	7/20/01
Residence: City	Mount Pleasant	**State**	SC	**Country**	US	**Citizenship**	US
Post Office Address	1010 Caseque Province, Mount Pleasant, SC 29464						
Post Office Address							
City	Mount Pleasant	**State**	SC	**ZIP**	29464	**Country**	US

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle (if any)			**Family Name or Surname**				
Inventor's Signature						**Date**	
City		**State**		**Country**		**Citizenship**	
Post Office Address							
Post Office Address							
City		**State**		**ZIP**		**Country**	

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle (if any)			**Family Name or Surname**				
Inventor's Signature						**Date**	
City		**State**		**Country**		**Citizenship**	
Post Office Address							
Post Office Address							
City		**State**		**ZIP**		**Country**	

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